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**14. ABSTRACT**

Recent molecular genetic studies suggest a four-hit hypothesis involving inactivation of both the *INI1/SNF5* and *NF2* tumor suppressor genes in the formation of schwannomatosis-associated tumors. To generate a mouse model for schwannomatosis, we have proposed to simultaneously inactivate both *Ini1/Snf5* and *Nf2* in *NF2*-expressed tissues. Toward achieving this goal, we have produced five lines of transgenic *NF2CreER* mice carrying a 2.4-kb *NF2* promoter driven a CreER expression unit. To prove the utility of the inducible CreER system, we generated *NestinCreER*;$Nf2^{flox2/flox2}$ mice. By tamoxifen injection, we inactivated *Nf2* in neuroprogenitor cells at various times during embryonic development. We found that *Nf2* is essential for neurulation and neuroepithelial progenitor proliferation during mammalian brain development. In contrast, mice with *Nf2* inactivation during mid-to-late gestation efficiently developed schwannomas, suggesting that *NestinCreER*;$Nf2^{flox2/flox2}$ mice may serve as an alternative model for NF2-associated schwannomas. By mating *NF2CreER* mice with $Nf2^{flox2/flox2}$ mice, compound *NF2CreER*;$Nf2^{flox2/flox2}$ mice were produced. Like *NestinCreER*;$Nf2^{flox2/flox2}$ mice with *Nf2* inactivation during mid-to-late gestation, an *NF2CreER*;$Nf2^{flox2/flox2}$ mouse developed a schwannoma at about one year of age, suggesting that this mouse line expresses functional CreER activity. In addition, by mating *NF2CreER* mice with $Snf5^{flox/flox}$ mice, we generated *NF2CreER*;$Snf5^{flox/flox}$ mice for further analysis.

**15. SUBJECT TERMS**
Schwannomatosis, neurofibromatosis type 2 (NF2), *NF2* and *INI1/SNF5* tumor suppressor genes, vestibular schwannomas, malignant rhabdoid tumors (MRTs), merlin, *NF2* promoter, conditional inducible knockout, tamoxifen, CreER, neural tube defects, neuroprogenitors

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## Introduction

Schwannomatosis is a newly recognized classification of neurofibromatoses (NF). It shares many features of the NF; however, patients with schwannomatosis develop multiple schwannomas in cranial, spinal, and peripheral nerves but do not have vestibular tumors seen in neurofibromatosis type 2 (NF2) or neurofibromas seen in neurofibromatosis type 1 (NF1) (MacCollin et al., 2005). At the current time, the cause for schwannomatosis is largely unknown, and no medical treatment or drug is available for schwannomatosis-associated tumors. Identification of novel therapeutics for the treatment of schwannomatosis is urgently needed and requires a disease-specific animal model for preclinical drug testing. Recent molecular genetic studies suggest a four-hit hypothesis involving inactivation of both the *INI1/SNF5* and *NF2* tumor suppressor genes in the formation of schwannomatosis-associated tumors (Hulsebos et al., 2007; Sestini et al., 2008; Hadfield et al., 2008). The **objective** of this study is to generate a mouse model for schwannomatosis by simultaneously inactivating both the mouse *Ini1/Snf5* and *Nf2* genes in specifically affected tissues, including Schwann cells.

Previous studies show that mutations in the *NF2* gene are frequently found in NF2-associated vestibular schwannomas. Mice with conditional *Nf2* inactivation in Schwann cells using the myelin protein P0 promoter-driven Cre (*P0Cre*) driver display some characteristics of NF2, including Schwann cell hyperplasia and schwannomas. However, the schwannomas were seen only in a small fraction of *P0Cre*;$Nf2^{flox2/flox2}$ mice after 10 months of age (Giovannini et al., 2000). Similarly, specific inactivating mutations in *SNF5* have been identified in the majority of human malignant rhabdoid tumors (MRTs). Mice heterozygous for *Snf5* developed tumors consistent with MRTs beginning as early as 5 weeks of age (Roberts et al., 2000, 2002). Occasionally, some $Snf5^{+/-}$ heterozygous mice also have spinal schwannomas (Isakoff et al., 2005). Thus, it will be interesting to see if simultaneous inactivation of both *Snf5* and *Nf2* will result in efficient schwannoma formation.

To achieve this goal, we have designed a conditional gene knockout approach using the regulatory sequence of the *NF2* gene to achieve target gene inactivation. We plan to generate transgenic mice carrying the 2.4-kb *NF2* promoter–driven *Cre* gene (*NF2Cre*). Previously, we showed that the *NF2* promoter could direct transgene expression to various NF2-affected tissues, including acoustic ganglion, trigeminal ganglion, spinal ganglia, the ependymal cell-containing tela choroidea, and the retinal pigmented epithelium (Akhmametyeva et al., 2006). As an alternative approach, we will use the inducible CreER system (Danielian et al., 1998) or another Schwann cell-specific promoter to generate the gene knockout. To simultaneously inactivate *Snf5* and *Nf2* in NF2-affected tissues, we will cross *NF2Cre* mice with $Nf2^{flox2/flox2}$ and $Snf5^{flox/flox}$ mice (Giovannini et al., 2000; Roberts et al., 2002). Triple compound *NF2Cre*;$Nf2^{flox2/flox2}$;$Snf5^{flox/flox}$ mice will be obtained and closely monitored for any abnormalities, including tumor formation. Any tumors developed in these mice will be excised and used in immunohistochemical analysis for tumor type, proliferation index, and protein expression.

If *NF2Cre*;$Nf2^{flox2/flox2}$;$Snf5^{flox/flox}$ mice efficiently develop multiple schwannomas, the study would provide direct proof of the involvement of both the *INI1/SNF5* and *NF2* genes in the genesis of schwannomatosis-associated tumors. The availability of such an animal model will allow us not only to better understand the synergistic role of *Ini1/Snf5* and *Nf2* in schwannomatosis tumorigenesis, but also to perform preclinical drug testing, ultimately leading to a cure of this difficult and debilitating disease.

**Body**

Aim 1: Production of transgenic *NF2Cre* (or *CreER*) mice

Task 1. To simultaneously inactivate *Snf5* and *Nf2* in specifically affected tissues, including Schwann cells, we decided to generate transgenic *NF2CreER* mice carrying a 2.4-kb *NF2* promoter-driven a tamoxifenized Cre (or CreER, the Cre recombinase fused with the tamoxifen-specific ligand-binding domain of estrogen receptor) expression unit. The reason why we generated *NF2CreER*, instead of *NF2Cre*, is because the CreER system allows one to induce time-controllable gene inactivation (Danielian et al., 1998). Since we have shown that the *NF2* promoter is expressed during early development (Akhmametyeva et al., 2006) and since both the *Nf2* and *Snf5* functions are essential during embryonic development (McClatchey et al., 1997; Roberts et al., 2000), conditional inactivation of both *Nf2* and *Snf5* using *NF2Cre* is likely to result in embryonic lethality.

To prove the utility of the inducible CreER system for gene inactivation, we first generated *NestinCreER*;$Nf2^{flox2/flox2}$ mice by crossing $Nf2^{flox2/flox2}$ mice (Giovannini et al., 2000; kindly provided to us by Dr. Marco Giovannini at House Ear Institute) with a transgenic *NestinCreER* line, which carries a CreER expression unit under the control of the second-intron enhancer element of the rat *Nestin* gene. The *NestinCreER* line has been shown to induce time-controllable Cre-loxP recombination in neuroprogenitor cells (Burns et al., 2007). By administration of tamoxifen into pregnant *NestinCreER*;$Nf2^{flox2/flox2}$ mice, we inactivated *Nf2* in neuroprogenitor cells at various times during embryonic development (Figure 1). We found that *Nf2* inactivation in neuroprogenitor cells (referred to as $Nf2^{Nestin}$CKO) during early gestation causes defects in neural tube closure and disrupts the apical adherens junctions. Surprisingly, these alterations in cell-cell contact abolish multiple mitogenic signaling pathways in the ventricular zone, leading to a marked reduction of the progenitor pool with moderately increased apoptosis. In contrast, mice with *Nf2* inactivation during mid-to-late gestation developed schwannomas and lymphomas at a high frequency. In addition to activation of the receptor-mediated MAPK and AKT pathways, these $Nf2^{-/-}$ mouse schwannoma cells show abundant β-catenin and readily express its downstream nuclear signals, including Tcf1, cyclin $D_1$, and c-Myc. Activation of β-catenin's downstream signals was confirmed in human vestibular schwannomas, suggesting that merlin-deficient schwannoma cells acquire a mechanism to activate the β-catenin signaling pathway. Collectively, these results indicate that the *Nf2* protein merlin either inhibits or supports cell proliferation dependent on the biological context. The $Nf2^{Nestin}$CKO mice that we have generated may serve as an alternative model for NF2-associated schwannomas (Akhmametyeva et al., 2010; see attached Manuscript).

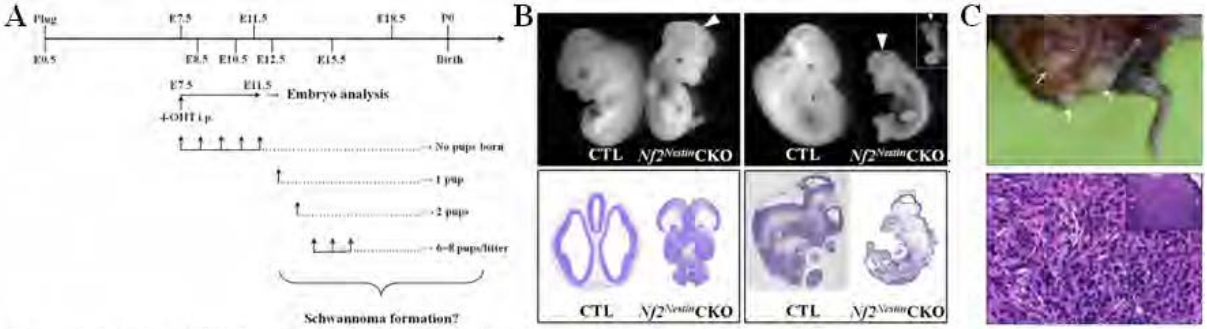


*Figure 1.* Analysis of *Nf2* function during neural tube development and tumorigenesis. (A) The timeline of tamoxifen (4-OHT) injection into pregnant *Nestin-CreER;*$Nf2^{flox2/flox2}$ mice to inactivate *Nf2*. (B) *Nf2* inactivation in neuroprogenitor cells during early gestation resulted in defects in neural tube closure (arrowheads), such as exencephaly. CTL, control embryo without tamoxifen injection. (C) An $Nf2^{Nestin}$*CKO* mouse with *Nf2* inactivation at E14.5 developed multiple schwannomas (arrows) at one year of age (top panel). Hematoxylin and eosin staining revealed typical spindle-to-round schwannoma cells with pleomorphic nuclei (bottom panel).

As we have successfully utilized the CreER system to study *Nf2* function during development and tumorigenesis, we subsequently generated the aforementioned *NF2-CreER* construct and used it to generate five lines of transgenic mice. These transgenic *NF2CreER* mice are normal and fertile and have been bred with wild-type FVB/N mice to produce offspring for the following tasks.

Task 2. To examine whether the transgenic *NF2CreER* mice express functional CreER activity, we crossed all five *NF2CreER* lines with $Nf2^{flox2/flox2}$ mice and an enhanced green fluorescence protein (EGFP) Reporter (also called CAG-CAT-EGFP) line, which carries the *loxP-STOP-loxP-EGFP* allele under the control of the β-actin promoter fused with a cytomegalovirus immediate-early gene enhancer (Nakamura et al., 2006). If CreER activity is expressed, compound *NF2CreER*;$Nf2^{flox2/flox2}$ or *NF2CreER*;EGFP Reporter mice, when injected with tamoxifen, should allow us to inactivate *Nf2* or express EGFP in *NF2*-expressed tissues, respectively. Interestingly, the *NF2CreER*;$Nf2^{flox2/flox2}$ line 4837 developed a schwannoma at about one year of age (Figure 2), similar to those observed in the $Nf2^{Nestin}$CKO mice with *Nf2* inactivation during mid-to-late gestation. These results suggest that this *NF2CreER*;$Nf2^{flox2/flox2}$ mouse line expresses functional CreER activity.

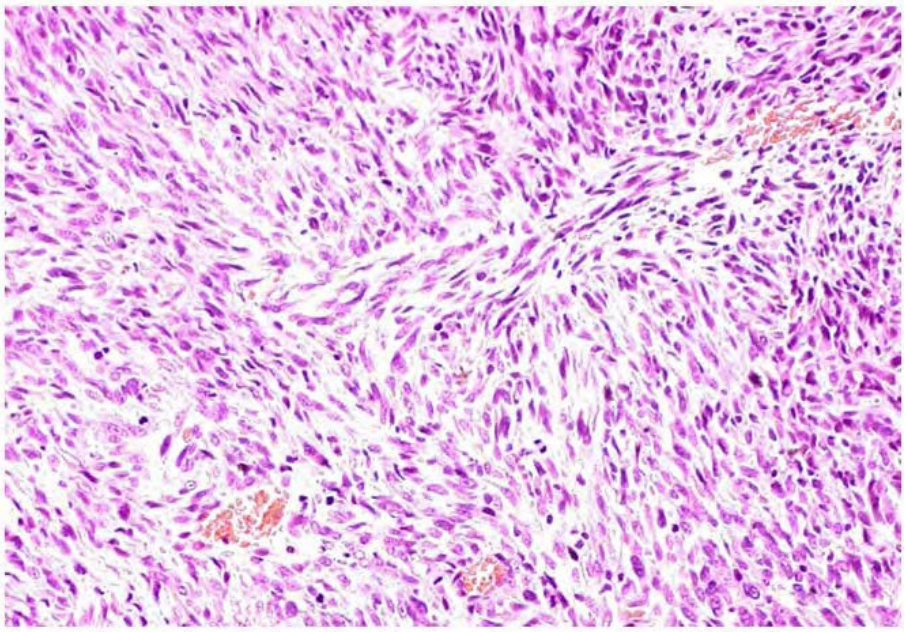

*Figure 2.* Hematoxylin and eosin staining of a schwannoma developed in the hind leg of an *NF2CreER*;$Nf2^{flox2/flox2}$ mouse at about one year of age shows spindle-shaped tumor cells.

Aim 2: <u>Conditional inactivation of both the *Snf5* and *Nf2* tumor suppressor genes in NF2-affected tissues</u>

Task 3. To generate conditional *Snf5* knockout, we also obtained mice carrying a floxed ($Snf5^{flox}$), inverted ($Snf5^{inv}$), or heterozygous ($Snf5^{+/-}$) allele of *Snf5* from Dr. Charles Roberts at the Dana-Farber Cancer Institute (Roberts et al., 2002). We confirmed that mice carrying a heterozygous allele of *Snf5* developed malignant rhabdoid tumors. Figure 3 shows that the mouse MRT tumor cells display characteristic eccentric nuclei and prominent nucleoli with some tumor cells containing multiple nuclei. By intercrossing $Snf5^{flox/+}$ mice, we generated $Snf5^{flox/flox}$ mice, which were further mated with *NF2CreER* mice to produce *NF2CreER*;$Snf5^{flox/flox}$ mice. Using these mice, we are presently examining the effect of *Snf5* inactivation in *NF2*-expressed tissues.

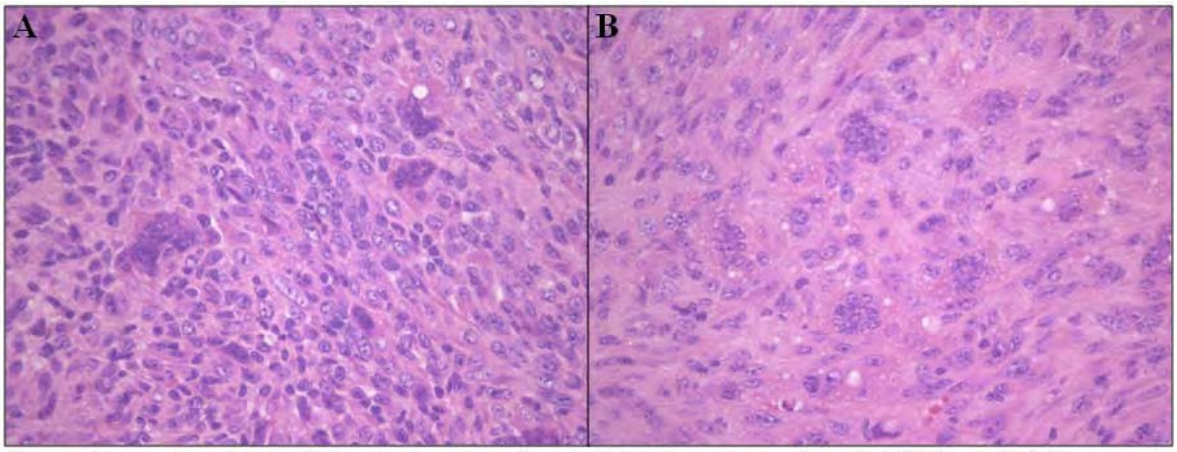


*Figure 3.* Hematoxylin and eosin staining of sections of a malignant rhabdoid tumor developed in an $Snf5^{+/-}$ (A) or $Snf5^{Inv/-}$ (B) mouse. Note that the tumor cells are characterized by eccentric nuclei, prominent nucleoli and eosinophilic cytoplasmic inclusions. Some tumor cells contain multiple nuclei.

Task 4. To simultaneously inactivate *Nf2* and *Snf5*, we will cross *NF2CreER*;$Snf5^{flox/flox}$ mice with $Nf2^{flox2/flox2}$ mice to generate triple compound *NF2CreER*;$Snf5^{flox/flox}$;$Nf2^{flox2/+}$ and *NF2CreER*;$Snf5^{flox/flox}$;$Nf2^{flox2/flox2}$ mice. Alternatively, we can use *NF2CreER*;$Nf2^{flox2/flox2}$ mice to cross with $Snf5^{flox/flox}$ mice. Due to our Material Transfer Agreement with Dr. Marco Giovannini for the $Nf2^{flox2/flox2}$ mice, we will perform this experiment in collaboration with his group. We are in the process of consulting with Dr. Giovannini in regards to the execution of this experiment.

Task 5. Once the *NF2CreER*;$Snf5^{flox/flox}$;$Nf2^{flox2/+}$ and *NF2*Cre;$Nf2^{flox2/flox2}$;$Snf5^{flox/flox}$ mice are generated, they will be closely watched for any abnormalities, including tumor formation.

**Key Research Accomplishments**

(1) To conditionally inactivate *Nf2* and *Snf5* in *NF2*-affected tissues, we generated five lines of *NF2CreER* mice. At least one of the *NF2CreER* lines has been shown to express functional CreER activity.

(2) We validated the utility of the CreER system by generating *NestinCreER*;$Nf2^{flox2/flox2}$ mice. By tamoxifen injection into pregnant *NestinCreER*;$Nf2^{flox2/flox2}$ mice, we show that *Nf2* is essential for neurulation and neuroepithelial progenitor proliferation during mammalian brain development. In contrast, mice with *Nf2* inactivation during mid-to-late gestation developed schwannomas and lymphomas at a high frequency. Like human vestibular schwannomas, the merlin-deficient mouse schwannoma cells display activation of multiple growth signaling pathways. Collectively, our results indicate that merlin either inhibits or supports cell proliferation dependent on the biological context. The $Nf2^{Nestin}$CKO mice that we have generated may serve as an alternative model for NF2-associated schwannomas.

(3) By mating *NF2CreER* mice with $Nf2^{flox2/flox2}$ mice, compound *NF2CreER*;$Nf2^{flox2/flox2}$ mice were produced. Like *NestinCreER*;$Nf2^{flox2/flox2}$ mice with *Nf2* inactivation during mid-to-late gestation, an *NF2CreER*;$Nf2^{flox2/flox2}$ mouse developed a schwannoma at about one year of age. Also, by mating *NF2CreER* mice with $Snf5^{flox/flox}$ mice, we generated *NF2CreER*;$Snf5^{flox/flox}$ mice.

**Reportable Outcomes**

During this grant period, we presented three research abstracts to The 2010 NF Conference held at Baltimore, MD. A manuscript describing the role of the *Nf2* gene during neural tube development and tumorigenesis was prepared and submitted to EMBO Journal for consideration. We acknowledged the support from the Department of Defense Neurofibromatosis Research Programs in all of these publications. Summaries of the abstracts and manuscripts are provided below.

Abstracts

(1) Akhmametyeva, E.M., J. Huang, C.-Y. Kuan, D.B. Welling, L.-S. Chang. 2010. Merlin Is Essential for Neurulation and Neuroepithelial Progenitor Proliferation in Mammalian Brain Development. This abstract was selected for a platform presentation at The 2010 NF Conference, Baltimore, MD.

By generating *NestinCreER*;*Nf2*$^{flox2/flox2}$ mice, we analyzed merlin function during development and tumorigenesis. We showed that merlin is essential for neurulation and neuroepithelial progenitor proliferation during mammalian brain development. In addition, we found that mice with *Nf2* inactivation during mid-to-late gestation developed schwannomas and/or lymphomas at a high frequency. Collectively, our results indicate that merlin either inhibits or supports cell proliferation dependent on the biological context.

(2) Huang, J., E.M. Akhmametyeva, S.S. Burns, M. Giovannini, D.B. Welling, and L.-S. Chang. 2010. Merlin Is Required for Proliferation of Mammary Epithelial Cells during Mammary Gland Development. This abstract was selected for a poster presentation at The 2010 NF Conference, Baltimore, MD.

To examine whether merlin has pro-proliferation functions in another developmental context, we conditionally inactivated *Nf2* during different stages of mammary gland development using three mammary epithelial cell-specific Cre lines, the mouse mammary tumor virus (*MMTV*)-*Cre*, the whey acidic protein (*WAP*)-*Cre*, and the β-lactoglobulin (*BLG*)-*Cre* lines. We showed that merlin plays an important role in mammary epithelial cell proliferation. *Nf2* inactivation in luminal epithelial cells during pregnancy resulted in markedly decreased lobuloalveolar morphogenesis. Our results indicate that merlin has pro-proliferation roles in mammary epithelial cells.

(3) Oblinger, J.L., T. Lee, M. Packer, J. Huang, M.L. Bush, S.K. Kulp, C.-S. Chen, M. Giovannini, D.B. Welling, A. Jacob, and L.-S. Chang. 2010. HDAC42 and OSU-03012, Novel Small-Molecule Inhibitors for the Treatment of Vestibular Schwannomas. This abstract was selected for a platform presentation at The 2010 NF Conference, Baltimore, MD.

In an attempt to develop novel medical therapies for NF2-associated vestibular schwannomas, we tested the growth inhibitory and anti-tumor activities of OSU-03012 and HDAC42, two small molecule inhibitors of the AKT pathway, which is frequently activated in vestibular schwannomas. We show that both OSU-03012 and HDAC42 potently decrease schwannoma cell proliferation. OSU-03012 selectively induces apoptosis in vestibular schwannoma and HMS-97 malignant schwannoma cells at doses that spare normal Schwann cells through the mitochondria-dependent intrinsic pathway. Akt phosphorylation was greatly reduced in schwannoma cells treated with either OSU-03012 or HDAC42. In addition, both

OSU-03012 and HDAC42 reduced the size of HMS-97 xenograft tumors in SCID mice. Data from these preclinical models will ultimately assist in the design of Phase 1 clinical trials on NF2-associated vestibular schwannomas and malignant schwannomas.

Manuscript Submitted

(1) Akhmametyeva, E.M., J. Huang, C,-Y. Kuan, D.B. Welling, and L.-S. Chang. 2010. Merlin Can Either Support or Inhibit Cell Proliferation Depending on the Biological Context. Submitted to EMBO J.

To better understand how merlin participates in regulating neural tube development, we used two lines of Cre-drivers for conditional *Nf2* gene deletion: (1) the *Wnt1-Cre* line (Danielian et al., 1998), which introduces Cre-LoxP recombination in the midbrain and the dorsal neural tube from E8.5, and (2) a tamoxifen-inducible *Nestin-CreER* line (Burns et al., 2007), which uses a rat second-intron enhancer element of the *Nestin* gene to induce time-controllable Cre-loxP recombination in neuroepithelial progenitors at different stages of embryogenesis. We show that *Nf2* inactivation in the dorsal neural tube using the *Wnt1-Cre* line impairs neural tube closure and neural crest cell formation and migration. We also demonstrate that *Nf2* inactivation in neuroprogenitor cells (referred to as $Nf2^{Nestin}$CKO) during early gestation using an inducible *Nestin-CreER* line causes defects in neural tube closure and disrupts the apical adherens junctions. Surprisingly, these alterations in cell-cell contact abolish multiple mitogenic signaling pathways in the ventricular zone, leading to a marked reduction of the progenitor pool with moderately increased apoptosis. In contrast, mice with *Nf2* inactivation during mid-to-late gestation developed schwannomas and lymphomas at a high frequency. In addition to activation of the receptor-mediated MAPK and AKT pathways, these $Nf2^{-/-}$ mouse schwannoma cells show abundant β-catenin and readily express its downstream nuclear signals, including Tcf1, cyclin $D_1$, and c-Myc. Activation of β-catenin's downstream signals was confirmed in human vestibular schwannomas, suggesting that merlin-deficient schwannoma cells acquire a mechanism to activate the β-catenin signaling pathway. Collectively, our results uncover important pro-proliferation functions for merlin during early brain development, which contrast with its anti-proliferative roles in tumor suppression. The $Nf2^{Nestin}$CKO mice that we have generated may serve as an alternative model for NF2-associated schwannomas.

**Conclusion**

To generate a mouse model for schwannomatosis, we have proposed to simultaneously inactivate both the *Ini1/Snf5* and *Nf2* tumor suppressor genes in *NF2*-expressed tissues. Toward achieving this goal, we have produced five lines of transgenic *NF2CreER* mice carrying a CreER-expression unit driven under the control of the regulatory sequence of the *NF2* gene. To prove the utility of the inducible CreER system, we generated *NestinCreER*;$Nf2^{flox2/flox2}$ mice. By administration of tamoxifen into pregnant *NestinCreER*;$Nf2^{flox2/flox2}$ mice, we inactivated *Nf2* in neuroprogenitor cells at various times during embryonic development. We found that *Nf2* is essential for neurulation and neuroepithelial progenitor proliferation during mammalian brain development. In contrast, mice with *Nf2* inactivation during mid-to-late gestation developed schwannomas and lymphomas at a high frequency. Like human vestibular schwannomas, the merlin-deficient mouse schwannoma cells display activation of multiple growth signaling pathways. Collectively, our results indicate that merlin either inhibits or supports cell proliferation dependent on the biological context. By mating *NF2CreER* mice with $Nf2^{flox2/flox2}$

mice, compound *NF2CreER*;*Nf2*$^{flox2/flox2}$ mice were produced. Like *NestinCreER*;*Nf2*$^{flox2/flox2}$ mice with *Nf2* inactivation during mid-to-late gestation, an *NF2CreER*;*Nf2*$^{flox2/flox2}$ mouse developed a schwannoma at about one year of age, suggesting that this mouse line expresses functional CreER activity. In addition, by mating *NF2CreER* mice with *Snf5*$^{flox/flox}$ mice, we generated *NF2CreER*;*Snf5*$^{flox/flox}$ mice. Using these *NF2CreER*;*Snf5*$^{flox/flox}$ and *NF2CreER*;*Nf2*$^{flox2/flox2}$ mice, we are in the process of setting up a collaboration with Dr. Giovannini to generate triple compound *NF2CreER*;*Snf5*$^{flox/flox}$;*Nf2*$^{flox2/+}$ and *NF2CreER*;*Snf5*$^{flox/flox}$;*Nf2*$^{flox2/flox2}$ mice for further analysis.

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**Appendices**

Three abstracts

(1) Akhmametyeva, E.M., J. Huang, C.-Y. Kuan, D.B. Welling, L.-S. Chang. 2010. Merlin Is Essential for Neurulation and Neuroepithelial Progenitor Proliferation in Mammalian Brain Development. The 2010 NF Conference, Baltimore, MD.

(2) Huang, J., E.M. Akhmametyeva, S.S. Burns, M. Giovannini, D.B. Welling, and L.-S. Chang. 2010. Merlin Is Required for Proliferation of Mammary Epithelial Cells during Mammary Gland Development. The 2010 NF Conference, Baltimore, MD.

(3) Oblinger, J.L., T. Lee, M. Packer, J. Huang, M.L. Bush, S.K. Kulp, C.-S. Chen, M. Giovannini, D.B. Welling, A. Jacob, and L.-S. Chang. 2010. HDAC42 and OSU-03012, Novel Small-Molecule Inhibitors for the Treatment of Vestibular Schwannomas. This abstract was selected for a platform presentation at The 2010 NF Conference, Baltimore, MD.

One submitted manuscript

(1) Akhmametyeva, E.M., J. Huang, C,-Y. Kuan, D.B. Welling, and L.-S. Chang. 2010. Merlin Can Either Support or Inhibit Cell Proliferation Depending on the Biological Context. Submitted to EMBO J.

*This abstract was selected for a platform presentation at The 2010 NF Conference at Baltimore, MD*

**Merlin Is Essential for Neurulation and Neuroepithelial Progenitor Proliferation in Mammalian Brain Development**

**Presenting author: Elena M. Akhmametyeva, MD, PhD**
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Merlin, the *Neurofibromatosis 2* (*Nf2*) gene product, mediates contact-dependent growth arrest in adult tissues, but its functions in the highly proliferative neural tube during embryogenesis are unclear. Here we show that *Nf2* inactivation in the dorsal neural tube using the *Wnt1-Cre* line impairs neural tube closure and neural crest cell formation and migration. We also demonstrate that *Nf2* inactivation in the early neuroepithelium using an inducible *Nestin-CreER* line causes defects in neural tube closure and disrupts the apical adherens junctions. Surprisingly, these alterations in cell-cell contact abolish multiple mitogenic signaling pathways in the ventricular zone, leading to a marked reduction of the progenitor pool with only moderately increased apoptosis. In contrast, mice with *Nf2* inactivation in neuroprogenitor cells during mid-to-late gestation developed schwannomas and lymphomas at a high frequency. In addition to activation of the receptor-mediated MAPK and AKT pathways, these mouse schwannoma cells show abundant β-catenin and readily express its downstream nuclear signals, including Tcf1, cyclin $D_1$, and c-Myc. Activation of the β-catenin pathway is confirmed in human vestibular schwannomas, suggesting that merlin-deficient schwannoma cells acquire a mechanism to activate β-catenin and its downstream targets. These results suggest that merlin either inhibits or supports cell proliferation dependent on the biological context. The *Nestin-CreER*;*Nf2*$^{flox2/flox2}$ mice that we have generated may serve as an alternative model for NF2-associated schwannomas.

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*This abstract was selected for a poster presentation at The 2010 NF Conference at Baltimore, MD*

**Merlin Is Required for Proliferation of Mammary Epithelial Cells during Mammary Gland Development**

**Presenting author: Jie Huang, MD, PhD**

*Nationwide Children's Hospital and The Ohio State University*

The *Neurofibromatosis 2* (*NF2*) gene, which encodes the tumor suppressor protein merlin, is frequently inactivated in neurofibromatosis type 2 (NF2)-associated vestibular schwannomas and meningiomas. Mice heterozygous for *Nf2* develop a range of metastatic tumors, and conditional biallelic inactivation of *Nf2* in Schwann cells or arachnoidal cells leads to NF2-like tumors. We previously showed that *Nf2* inactivation in neuroprogenitor cells during early development disrupts neuroepithelial cell integrity, abolishes the formation of apical adherens junctions and associated receptor tyrosine kinases, and impairs their downstream signals, consequently leading to depletion of the neuroprogenitor pool and neural tube defects. Although these results indicate that merlin is essential for neurulation and neuroprogenitor proliferation, they also identify an unprecedented role for merlin in promoting cell proliferation during mammalian brain development, which contrasts with its function as a tumor and metastatic suppressor in specific cell types, such as Schwann cells. To examine whether merlin has pro-proliferation functions in another developmental context, we conditionally inactivated *Nf2* during different stages of mammary gland development using three mammary epithelial cell-specific Cre lines, the mouse mammary tumor virus (*MMTV*)-*Cre*, the whey acidic protein (*WAP*)-*Cre*, and the β-lactoglobulin (*BLG*)-*Cre* lines. We showed that the *MMTV-Cre*-mediated excision of exon 2 of the *Nf2* gene led to embryonic lethality prior to embryonic day (E)12.5. Examination of mammary buds in E12.5 $Nf2^{MMTV}$-knockout embryos revealed defects in epithelial cells. Conditional deletion of *Nf2* in mammary luminal epithelial cells during mid-gestation of pregnancy using *WAP-Cre* resulted in decreased proliferation of both luminal epithelial and myoepithelial cells during successive gestation cycles, consequently leading to decreased milk production and malnourishment of the offspring by the second lactation. Immunostaining analysis revealed that luminal epithelial cells in the mammary glands of $Nf2^{Wap}$-knockout mice showed decreased E-cadherin, β-catenin, and GSK-3β expression and had no nuclear Tcf1. Similarly, mice with *Nf2* inactivation in luminal epithelial cells during early gestation using *BLG-Cre* displayed nursing difficulties due to markedly decreased lobuloalveolar morphogenesis, which were observed by the first lactation. In addition to decreased proliferation, $Nf2^{BLG}$-knockout luminal epithelial cells had reduced expression of estrogen receptor α and progesterone receptor in the nucleus. Together, these results indicate that merlin has pro-proliferation roles in mammary epithelial cells and further suggest that merlin may regulate nuclear receptor expression.

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*This abstract was selected for a platform presentation at The 2010 NF Conference at Baltimore, MD*

**HDAC42 and OSU-03012, Novel Small-Molecule Inhibitors for the Treatment of Vestibular Schwannomas**

**Presenting author: Janet L. Oblinger, PhD**
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Inactivating mutations in the *Neurofibromatosis 2* (*NF2*) gene, which encodes the tumor suppressor protein merlin, commonly cause vestibular schwannomas (VS). Current treatment options for VS are limited to either surgical resection or radiotherapy. However, serious post-treatment morbidities can occur, such as deafness, facial nerve paralysis, or radiation-induced malignant transformation. Presently, there are no accepted chemotherapeutic options for VS since these tumors are resistant to conventional chemotherapy. The Akt/PKB pathway has been strongly linked to the pathogenesis of many tumor types, including VS, and is considered as a promising target for drug therapy. OSU-03012 inhibits phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates and activates the pro-survival protein Akt. HDAC42 is a histone deacetylase (HDAC) inhibitor that inhibits Akt activation and other mitogenic signaling pathways. We show that both OSU-03012 and HDAC42 potently decrease schwannoma cell proliferation with $IC_{50}$ values in the low micromolar (OSU-03012) and high nanomolar (HDAC42) range. OSU-03012 selectively induces apoptosis in VS and HMS-97 schwannoma cells at doses that spare normal Schwann cells through the mitochondria-dependent intrinsic pathway. This antiproliferative effect correlates with a strong inhibition of critical pro-survival signaling pathways. Akt phosphorylation was greatly reduced in schwannoma cells treated with either OSU-03012 or HDAC42. In addition, VS cells treated with HDAC42 show markedly reduced Erk1/2 phosphorylation. Further, OSU-03012 reduced the size of HMS-97 xenograft tumors by ~55% as determined by small-animal MRI, and the tumor histology showed massive areas of necrosis and decreased phospho-AKT staining. Similarly, HMS-97 xenograft tumor volume was reduced by ~58% in mice fed with HDAC42, and VS-xenografted SCID mice fed with HDAC42 showed a 50% reduction in tumor size. Data from these preclinical models will ultimately assist in the design of Phase 1 clinical trials on NF2-associated VS and malignant schwannomas.

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**Merlin Can Either Support or Inhibit Cell Proliferation Depending on the Biological Context**

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Running Title: *Nf2* function during development and tumorigenesis

Key Words: *Neurofibromatosis 2* (*NF2*) gene, tamoxifen, neural tube defects, neural crest cell, β-catenin, and schwannoma

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**SUMMARY**

Merlin, the *Neurofibromatosis 2* (*Nf2*) gene product, mediates contact-dependent growth arrest in adult tissues, but its functions in the highly proliferative neural tube during embryogenesis are unclear. Also, whether the timing of *Nf2* inactivation in specific cell types is critical for tumor formation is not known. Here we show that *Nf2* inactivation in the dorsal neural tube using the *Wnt1-Cre* line impairs neural tube closure and neural crest cell formation and migration. We also demonstrate that *Nf2* inactivation in neuroprogenitor cells (referred to as $Nf2^{Nestin}$CKO) during early gestation using an inducible *Nestin-CreER* line causes defects in neural tube closure and disrupts the apical adherens junctions. Surprisingly, these alterations in cell-cell contact abolish multiple mitogenic signaling pathways in the ventricular zone, leading to a marked reduction of the progenitor pool with moderately increased apoptosis. In contrast, mice with *Nf2* inactivation during mid-to-late gestation developed schwannomas and lymphomas at a high frequency. In addition to activation of the receptor-mediated MAPK and AKT pathways, these $Nf2^{-/-}$ mouse schwannoma cells show abundant β-catenin and readily express its downstream nuclear signals, including Tcf1, cyclin $D_1$, and c-Myc. Activation of β-catenin's downstream signals was confirmed in human vestibular schwannomas, suggesting that merlin-deficient schwannoma cells acquire a mechanism to activate the β-catenin signaling pathway. Collectively, these results indicate that merlin either inhibits or supports cell proliferation dependent on the biological context. The $Nf2^{Nestin}$CKO mice that we have generated may serve as an alternative model for NF2-associated schwannomas.

## INTRODUCTION

The *Neurofibromatosis 2* (*NF2*) gene encodes a tumor suppressor protein named merlin or schwannomin (Trofatter et al., 1993; Rouleau et al., 1993), which is frequently inactivated in neurofibromatosis type 2 (NF2), an autosomal dominant disorder characterized by the development of vestibular schwannomas, meningiomas, ependymomas and spinal schwannomas (reviewed in McClatchey and Giovannini, 2005; Welling et al., 2008). Unlike other tumor suppressors, merlin does not have any catalytic or DNA-binding domains. It belongs to the ezrin-radixin-moesin (ERM) family of cytoskeleton-associated proteins; however, merlin is the only member of this family to possess tumor suppressor functions. In addition to frequent inactivation in NF2-associated tumors, conditional biallelic inactivation of *Nf2* in Schwann cells or arachnoidal cells in mice leads to NF2-like tumors (Giovannini et al., 2000; Kalamarides et al., 2002), and mice heterozygous for *Nf2* develop a range of metastatic tumors exhibiting loss of heterozygosity of the remaining *Nf2* allele (McClatchey et al., 1998). In addition, merlin mediates contact-dependent inhibition of proliferation in confluent cells by restraining the membrane levels of multiple pro-mitogenic receptors, including epidermal growth factor receptor (EGFR) and ErbB2/3 (Curto et al., 2007; Lallemand et al., 2009).

Merlin also has important developmental functions. *Nf2*$^{-/-}$ mouse embryos die around embryonic day 7 (E7), exhibiting disorganized extraembryonic ectoderm (McClatchey et al., 1997). However, the functions of merlin in the embryo proper remain unclear, and it is not known whether merlin plays any roles at later stages of development. This is a particularly intriguing question because gene expression studies using either transgenic reporter mice or *in-situ* hybridization analysis show that merlin is enriched in the neural tube between E8.5 to 9.5 before it becomes more widely expressed in older embryos (Akhmametyeva et al., 2006). In

addition, a transient change in *NF2* promoter expression during neural tube closure, neural crest cell (NCC) migration, and tissue fusion has been observed, suggesting a role for merlin during these processes (Akhmametyeva et al., 2006; McLaughlin et al., 2007). Using paternally-inherited *Nestin-Cre* (*NesCre1*$^{P}$) mice, in which Cre-mediated gene-deletion gradually occurs from E8.5 to E18.5, McLaughlin et al. (2007) suggested that merlin is needed for the assembly of adherens junctions (AJs) in the developing neuroepithelium. The consequences of local *Nf2* deficiency are abnormal cell-cell adhesion and detachment–induced apoptosis (anoikis) during tissue fusion. However, the mosaic and asynchronous onset of gene-deletion with the *NesCre1*$^{P}$ mice may conceal important functions of merlin during mammalian brain development. Furthermore, this study precludes the analysis of merlin function in tumorigenesis because the mutant embryos died before birth.

To better understand how merlin participates in regulating neural tube development, we used two lines of Cre-drivers for conditional *Nf2* gene deletion: (1) the *Wnt1-Cre* line (Danielian et al., 1998), which introduces Cre-LoxP recombination in the midbrain and the dorsal neural tube from E8.5, and (2) a tamoxifen-inducible *Nestin-CreER* line (Burns et al., 2007), which uses a rat second-intron enhancer element of the *Nestin* gene to induce time-controllable Cre-loxP recombination in neuroepithelial progenitors at different stages of embryogenesis. In addition, we investigated the signaling mechanisms that are affected by merlin loss during development and tumorigenesis. Our results uncover important pro-proliferation functions for merlin during early brain development, which contrast with its anti-proliferative roles in tumor suppression.

## EXPERIMENTAL PROCEDURES

*Transgenic mice.* Various transgenic lines used in this study have been previously described.

The *Wnt1-Cre* line carries a *Cre* cDNA under the control of the *Wnt1* promoter and enhancer (Danielian et al, 1998). The *Nestin-CreER* line expresses a tamoxifen-inducible Cre (CreER) under the control of the *Nestin* enhancer/*hsp68* minimal promoter (Burns et al, 2007). The $Nf2^{flox2/flox2}$ line contains the floxed *Nf2* allele with exon 2 flanked by two *loxP* sites (Giovannini et al, 2000). The Rosa26 reporter line harbors the *loxP-STOP-loxP-LacZ* allele driven by the constitutive Rosa26 promoter (Soriano, 1999). The EGFP Reporter (also called CAG-CAT-EGFP) line carries the *loxP-STOP-loxP-EGFP* allele under the control of the β-actin promoter fused with a cytomegalovirus immediate-early gene enhancer (Nakamura et al., 2006).

*Generation of Wnt1-Cre-directed EGFP, Wnt1-Cre-directed Nf2 knockout, and Nestin-CreER-directed Nf2 knockout mice and embryos. Wnt1-Cre* mice were mated with *EGFP* Reporter mice to derive the *Wnt1-Cre;EGFP* Reporter genotype. For embryo harvesting, the day when the vaginal plug was found, embryos were aged as 0.5 day p.c. Cre activity in *Wnt1-Cre;EGFP* embryos and pups was examined by visualizing green fluorescence using a Lightools 3D Pan-A-See System. Also, *Wnt1-Cre* mice were crossed with $Nf2^{flox2/flox2}$ mice, and the resulting *Wnt1-Cre;*$Nf2^{flox2/+}$ male offspring were further mated with $Nf2^{flox2/flox2}$ female mice. Embryos from E7.5 to birth were dissected from pregnant females and fixed in 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS; Invitrogen). Fixed embryos were incubated in 30% sucrose, immersed in Optimal Cutting Temperature medium (Miles) for 30 min, and flash-frozen in 2-methylbutane (Sigma) chilled with dry ice. Eight-micron sections were obtained using a Leica cryostat and processed for immunostaining. For genotyping, DNA extracted from the brain or tail of the embryo or the yolk sac was used in polymerase chain reaction analysis for Cre or the wild-type, floxed, and deleted alleles of *Nf2* (Danielian et al,

1998; Giovannini et al, 2000).

To generate the *Nestin-CreER;Nf2* $^{flox2/flox2}$ genotype, we crossbred *Nestin-CreER* mice with *Nf2*$^{flox2/flox2}$ mice. The *Nestin-CreER;Nf2*$^{flox2/flox2}$ mice were mated with each other, and pregnant females were injected intraperitoneally with a single dose of tamoxifen (5 mg per 40 g body weight; Sigma) at various days p.c. For embryo analysis, we injected tamoxifen at E7.5 ~ E8.5, the time period when neural tube closure begins, and collected embryos at E10.5 ~ E11.5. Harvested embryos were fixed in 4% paraformaldehyde and processed for paraffin-embedded tissue sectioning and immunohistochemical analysis. In addition, some pregnant mice injected with tamoxifen at E7.5 ~ E16.5 were allowed to deliver to term. Newborn mice were genotyped, and closely monitored for any abnormalities, including tumor formation. Any tumors developed in these mice and some normal tissues, such as sciatic nerves, were harvested, fixed in 10% neutral buffered formalin, and processed for immunohistochemical analysis. Also, the brains of some embryos, mouse tails, and tumor tissues were used for genotyping.

*Culture of mouse neural tube explants and lentiviral-mediated short-hairpin RNA (shRNA) targeting.* E9.5 control (*Nf2*$^{flox2/flox2}$) and *Wnt1-Cre;Nf2*$^{flox2/flox2}$ embryos were collected, and their dorsal neural tubes, spanning from the caudal hindbrain region to the rostral trunk level, were excised in Dulbecco's modified Eagle (DME) medium. Neural tube segments were treated with 200 μg/ml dispase for 15 min at room temperature, freed of the ectoderm and somites, and plated in fibronectin-coated dishes containing DME medium supplemented with 10% fetal bovine serum according to Rovasio et al. (1983). Explant cultures were maintained at 37°C in a humidified 5% $CO_2$ incubator with medium replaced every three days. Migration of NCCs from neural tube explants was visualized using a Leica DM-IRB inverted microscope.

MISSION® shRNA lentiviral transduction particles targeting mouse *Nf2* (clone ID: NM_020898 2-1623s1c1) were purchased from Sigma. The sequence of the shRNA for *Nf2* is CCGGCGAGCGTACAAGAGATGAGTTCTCGAGAACTCATCTCTTGTACGCTCGTTTTTG. Cultures of neural tube explants from E9.5 control embryos were mock-infected or infected with *Nf2* shRNA lentiviral particles overnight according to manufacturer's instruction. The following day, virus was aspirated and fresh growth medium was added and replenished every three days. Efficiency of merlin knockdown in mouse embryo fibroblasts using the indicated lentiviral shRNA clone was estimated to be about 90% by Western blot analysis using an anti-merlin antibody.

*Immunofluorescence analysis.* Frozen tissue sections were fixed for 20 min in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 and 30mM sucrose. After three washes with 1% BSA in PBS, sections were permeabilized for 5 min in 0.2% saponin, 0.03M sucrose, and 1% BSA, blocked for 15 min in 5% normal goat serum (NGS), and incubated for 60 min at RT with a primary antibody at an appropriate dilution. Following blocking for 15 min in NGS, an Alexa® Fluor-conjugated secondary antibody (Invitrogen) diluted in 1% BSA was added to tissue sections for 30 min at RT. Stained slides were washed, mounted with ProLong® Gold Antifade reagent with DAPI (Invitrogen), and viewed using a Zeiss Axioskop® epifluorescence microscope.

Paraffin sections were deparaffinized in xylene and rehydrated in descending alcohol solutions, followed by heating in 0.1M sodium citrate, pH 6.0 in a pressure cooker for 30 min. After cooling at RT, sections were washed in PBS, blocked in 10% NGS for 60 min at RT, and incubated with a primary antibody at 4°C overnight. After washing in 0.1% Tween 20® and 1%

BSA, an Alexa® Fluor-conjugated secondary antibody was added to sections for 1 hr at RT. Stained sections were washed, counterstained with DAPI (Vector), mounted in Immu-mount (Thermo), and viewed as above.

*Immunohistochemistry.* Paraffin sections were deparaffinized and rehydrated, followed by antigen retrieval as described above. Endogenous peroxidase activity was blocked using 3% $H_2O_2$ for 15 min. For rabbit polyclonal or monoclonal antibodies, sections were incubated in Super Block solution (ScyTek Lab) for 10 min. For mouse monoclonal antibodies, sections were incubated in Super Block solution for 10 min and then in Mouse-to-Mouse Blocking solution (ScyTek Lab) for 60 min. Sections were then incubated with a primary antibody for 1 hr at RT. After washing, an UltraTek® anti-polyvalent biotinylated secondary antibody (ScyTek Lab) was added to the sections for 10 min. Stained sections were incubated with UltraTek® HRP (ScyTek Lab) for 10 min, treated with an AEC substrate, counterstained with hematoxylin, mounted with Immu-mount, and viewed.

*List of antibodies.* Primary antibodies used in immunofluorescence analysis included rabbit anti-merlin (Santa Cruz; sc-331), mouse anti-Cre (Covance; MMS-106P), rabbit anti-phospho-Histone H3 (Epitomics; 1173-1), mouse anti-microtubule-associated protein 2 (Covance; 14832502), rabbit anti-neuronal class III β-tubulin (Covance; 14891801), rabbit anti-cleaved Caspase-3 (Cell Signaling; 9661), mouse anti-β-catenin (BD Transduction Labs; 610153), rabbit anti-N-cadherin (Epitomics; 2019-1), and mouse anti-Nestin (Santa Cruz; sc-G1406). Secondary antibodies used included goat anti-mouse Alexa® Fluor 488, goat anti-rabbit Alexa® Fluor 488, goat anti-mouse Alexa® Fluor 568, and goat anti-rabbit Alexa® Fluor 568 (Invitrogen). Primary

antibodies used in immunohistochemistry studies included rabbit anti-Ki67 (NeoMarkers; RM-9106), mouse anti-cyclin $D_1$ (Santa Cruz; sc-450), rabbit anti-phospho-AKT($Ser^{473}$) (Cell Signaling; 9277), mouse anti-phospho-tyrosine (Millipore; 05-321), and anti-epidermal growth factor receptor (Cell Signaling; 4407).

## RESULTS

***Wnt1-Cre*-mediated *Nf2* deletion impairs neurulation and NCC migration.** To better understand merlin's role during neural tube development, we first searched for an appropriate Cre-driver line in the early neural tube. We crossed *Wnt1-Cre* mice (Danielian et al., 1998) with a stop-floxed EGFP reporter line (Nakamura et al., 2006) to evaluate the timing of *Wnt1-Cre*-mediated recombination. In bi-transgenic embryos, intense green fluorescence was detected in the midbrain region at E8.5 and in the midbrain and the entire dorsal neural tube at E9.5 (Supplementary Fig. 1), suggesting that the *Wnt1-Cre* line is a suitable driver for *Nf2* deletion during neurulation. Thus, we crossed *Wnt1-Cre* mice with $Nf2^{flox2/flox2}$ mice (Giovannini et al., 2000) to achieve conditional deletion of *Nf2*. We found that *Wnt1-Cre;*$Nf2^{flox2/+}$ mice were normal and fertile. However, when *Wnt1-Cre;*$Nf2^{flox2/+}$ mice were mated with each other or with $Nf2^{flox2/flox2}$ mice, the *Wnt1-Cre;*$Nf2^{flox2/flox2}$ genotype was never detected in >300 pups. Further analysis of embryos derived from the mating of *Wnt1-Cre;*$Nf2^{flox2/+}$ male and $Nf2^{flox2/flox2}$ female mice identified very few *Wnt1-Cre;*$Nf2^{flox2/flox2}$ (referred to as $Nf2^{Wnt1}$CKO) embryos. The incidence was 1.6% (4/250) at E8.5, 1.57% (5/318) at E9.5, and 1.27% (4/315) at E10.5, far less than the expected Mendelian inheritance ratio (25%) and indicating that the majority of $Nf2^{Wnt1}$CKO embryos died prior to E8.5. In the few surviving E8.5 $Nf2^{Wnt1}$CKO embryos, comparison of the PCR genotyping results to those using embryonic sac tissues showed

significant Cre-mediated deletion at the floxed *Nf2* locus in the midbrain (Fig. 1A), thus validating the conditional gene-targeting approach.

Upon inspection, the E8.5 $Nf2^{Wnt1}$CKO embryos exhibited prominent defects in neural tube closure compared with control (CTL) embryos that were of either the *Wnt1-Cre;*$Nf2^{flox2/+}$ or *Wnt1-Cre* genotype. At E8.5, closure of the neural tube in CTL embryos had already occurred in the cervical spinal cord region and begun to proceed toward the rostral and caudal ends. In contrast, the midbrain and hindbrain neural folds and the caudal end of neural tubes in $Nf2^{Wnt1}$CKO embryos remained open (Fig. 1B). By E9.0, CTL embryos had completed neural tube closure and body-axis rotation from a U-shaped to a C-shaped body configuration. In contrast, $Nf2^{Wnt1}$CKO embryos were smaller and paler and remained in a U-shaped body axis with the neural folds unfused. Defects in neural tube closure were also evident in E9.5 $Nf2^{Wnt1}$CKO embryos.

Next, we used immunofluorescence analysis to examine the surviving E9.5 $Nf2^{Wnt1}$CKO embryos. While CTL embryos, either stage-matched (at E8.5 with an unfused neural tube) or age-matched (at E9.5 with a closed neural tube) displayed a band of Phalloidin+ F-actin in the inner apical surface of the neural tube, this structure was undetectable in $Nf2^{Wnt1}$CKO embryos (Fig. 2A-C). Also, both the staged-matched and age-matched CTL embryos had an active mitotic activity in the apical surface, as indicated by many phospho-Histone 3 (pH3)-positive cells, but this labeling was greatly reduced in $Nf2^{Wnt1}$CKO embryos (Fig. 2D-F). In addition, immunostaining of β-tubulin III (TuJ1), a marker for early neurons, indicated that the poorly-differentiated mutant neural tube contained fewer scattered neurons with shorter axon extensions. Also, the ventral commissure was not found, and cranial nerve ganglia and dorsal root ganglia did not form (Fig. 2I). These results suggest a defect in axonal guidance due to *Nf2* loss and

support the idea that merlin function is required for the development of the NCC-derived peripheral nervous system.

To further assess the requirement of merlin in NCC formation and migration, we cultured dorsal neural tube explants from E9.5 CTL and *Nf2*$^{Wnt1}$CKO embryos in fibronectin-coated dishes. Consistent with previous reports (Rovasio et al., 1983), cultures of dorsal neural tube explants from CTL embryos displayed typical NCC migration, proliferation, and differentiation, forming a layer of neuroepithelial cells with glial extensions growing on top of it and neurons migrating along glial fibers (Fig. 3A,B). In contrast, neural tube explants from *Nf2*$^{Wnt1}$CKO embryos either could not adhere to the substratum and remained floating in the culture medium (Fig. 3C) or exhibited weak adhesion to the substratum but were unable to proliferate or initiate migration (Fig. 3D), even when the explants were cultured for one week. To confirm merlin's role in NCC migration, we examined the effect of merlin knockdown in cultures of E9.5 CTL neural tube explants using lentiviral transduced shRNA targeting *Nf2*. Neural tube explant cultures infected with *Nf2* shRNA lentiviral particles displayed less migrating epithelial cells on the bottom layer but had numerous glial extensions forming spider web-like structures as compared to mock-infected cultures (compare Fig. 3G-H with Fig. 3E-F). Taken together, these results indicate that merlin is required for neural tube closure and NCC formation and migration.

***Nf2* inactivation using the inducible *Nestin-CreER* line also causes defects in neural tube closure.** To confirm the requirement of merlin for neural tube closure and to further examine its underlying mechanisms, we next crossed *Nf2*$^{flox2/flox2}$ mice with *Nestin-CreER* mice to achieve tamoxifen-inducible Cre-loxP recombination (Hayashi and McMahon, 2002). The *Nestin-CreER* mice have been used for fate-mapping and gene-mutation in Nestin-positive neuroepithelial

progenitors (Burns et al., 2007; Nagao et al., 2008). Since defects in neural tube closure in *Nf2*$^{Wnt1}$CKO embryos were seen as early as E8.5, we administered tamoxifen to pregnant *Nestin-CreER;Nf2*$^{flox2/flox2}$ mice at around 8 days post coitus (p.c.) and examined embryos at E10.5 to E11.5 (Fig. 4). Without tamoxifen injection, *Nestin-CreER;Nf2*$^{flox2/flox2}$ embryos (referred to as CTL) were healthy and indistinguishable from wild-type embryos. However, *Nf2* inactivation in neuroprogenitor cells by tamoxifen induction (referred to as *Nf2*$^{Nestin}$CKO) at this early embryonic time-point severely affected the development of the neural tube, branchial arches, and heart. Similar to *Nf2*$^{Wnt1}$CKO embryos, the majority of *Nf2*$^{Nestin}$CKO embryos displayed neural tube defects (NTDs), including exencephaly (Fig. 4A). Based on PCR amplification of the *Nf2*-flox and the *Nf2*-deleted (Δ) alleles in DNA extracted from E10.5 brains, we estimated that the efficiency of *Nestin-CreER*-mediated recombination was about 60~80% at this stage (Fig. 4H). Also, NTDs were not observed in embryos obtained from pregnant *Nestin-CreER; ROSA26R* β-gal reporter mice receiving the same tamoxifen treatment (data not shown), eliminating the possibility that they were caused by the potential toxic effect of tamoxifen.

At E11.5, the rostral neural tubes in CTL embryos had already developed into a three-vesicle structure, consisting of the forebrain, midbrain, and hindbrain (Fig. 4B,C), with a tightly-organized neuroprogenitor cell layer in the apical/ventricular surface and a layer of differentiated neurons near the basal/pial surface (Fig. 4D). In contrast, the unclosed neural tubes in *Nf2*$^{Nestin}$CKO embryos were thinner and contained fewer, loosely-organized cells (Fig. 4E). Note that due to defects in neural tube closure, the apical surface and the basal lamina in the *Nf2*$^{Nestin}$CKO neural tube were situated opposite from those seen in the CTL neural tube, as clearly illustrated by TuJ1 staining for neuronal β-tubulin III. A uniform layer of TuJ1-positive neurons located superficial to the ventricular zone (VZ) was detected in neural tubes of CTL

embryos at E11.5 (Fig. 4F) or earlier embryonic time points E8.5 and E9.5 (Fig. 2G,H). However, a diffuse layer of labeled neurons, many of which scattered into the presumptive VZ region, was seen in the E11.5 *Nf2*$^{Nestin}$CKO neuroepithelium (Fig. 4G and Supplementary Fig. S2). Also, we observed that both cranial ganglia, such as trigeminal ganglia, and spinal ganglia did not develop properly in *Nf2*$^{Nestin}$CKO embryos (Supplementary Fig. S3). In conjunction with the findings from *Nf2*$^{Wnt1}$CKO embryos, these results indicate that loss of merlin affects neuronal differentiation.

Since neural tube development involves cell apoptosis (Oppenheim, 1991), we stained embryo sections for cleaved Caspase 3 (CC3), an activated form of Caspase 3 that serves as a marker for apoptosis. As reported previously (Kuan et al., 2000), we detected very few CC3-positive cells in CTL neural tubes at E11.5 (Fig. 4F) or earlier embryonic time points E8.5 and E9.5 (Supplementary Fig. S2). Intriguingly, we observed a moderate increase in CC3-positive cells in E11.5 *Nf2*$^{Nestin}$CKO neural tubes with some clustered in the non-proliferative zone of the mutant neuroepithelium (Fig. 4G and Supplementary Fig. S2), suggesting that *Nf2* inactivation in neuroprogenitor cells induce some but not massive apoptosis.

To confirm that the NTDs were caused by *Nf2* inactivation, we stained CTL and *Nf2*$^{Nestin}$CKO embryo sections for merlin and Cre. While intense merlin staining was detected throughout the E11.5 CTL neural tube, the *Nf2*$^{Nestin}$CKO neuroepithelium expressed substantially reduced merlin (Fig. 4J,K), and this decrease in merlin staining coincided with Cre protein expression in the mutant neuroepithelial cells (Fig. 4I). Together with the findings in *Nf2*$^{Wnt1}$CKO embryos and a previous report (McLaughlin et al., 2007), these results implicate a critical role of merlin in neurulation during mammalian brain development. Since the extent of apoptosis found in the *Nf2*$^{Nestin}$CKO neural tube is not dramatic, these results suggest that *Nf2*

inactivation may have an inhibitory effect on neuroprogenitor proliferation.

**Merlin is required for apical AJ formation in the neuroepithelium.** During brain development, apical neuroepithelial progenitors in the ventricular surface are joined by tight junctions and AJ complexes that contain N-cadherin and β–catenin as two key components (Rudini and Dejana, 2008). Several lines of evidence suggest that AJ-associated proteins regulate progenitor proliferation and differentiation (Farkas and Huttner, 2008). Since *Nf2* deficiency destabilizes AJs in cultured epithelial cells (Lallemand et al., 2003), we examined whether *Nf2* inactivation affected AJ formation in neuroepithelial progenitors.

As previously reported (Chenn et al., 1998; Aaku-Saraste et al., 2006), we detected a high concentration of N-cadherin and β-catenin in the honeycomb-like structures on the apical surface of E11.5 CTL neural tubes (Fig. 5A,C). Also, cortical N-cadherin and β-catenin staining extended to the developing cortical plate between neuroepithelial cells. Similar patterns of N-cadherin and β-catenin staining were also observed in E8.5 and E9.5 CTL neural tubes (Supplementary Fig. S4). In contrast, the apical honeycomb-like structures of N-cadherin and β-catenin were greatly diminished in E11.5 $Nf2^{Nestin}$CKO neural tubes, and little cortical N-cadherin and β-catenin staining was found in the poorly-organized mutant neuroepithelial cells (Fig. 5B,D). Since N-cadherin and β-catenin are key anchoring proteins for many cell-cell contact-associated signaling pathways (Rudini and Dejana, 2008), their absence on the apical surface of the $Nf2^{Nestin}$CKO neuroepithelium may alter downstream signal transduction events. Consistent with this notion, while phospho-Jun kinase (p-JNK) was concentrated on the apical surface, and to a lesser extent, on the basal side of CTL neural tubes (Fig. 6A), it was detected only on the basal side of $Nf2^{Nestin}$CKO neural tubes (Fig. 6D). Importantly, double-labeling with

TuJ1 on the same sections showed intact expression of this neuronal marker in *Nf2*$^{Nestin}$CKO embryos compared with CTL embryos (Fig. 6B-C,E-F). These results suggest that the reduction of p-JNK staining on the apical surface of the *Nf2*$^{Nestin}$CKO neural tube is likely due to defects in specific signaling mechanisms rather than poor vitality of the mutant tissue.

***Nf2* inactivation impairs mitotic signaling and depletes neuroepithelial progenitors.** Since apical junctional complexes in the neuroepithelium are enriched in signaling molecules (Woods and Bryant, 1993; Chenn et al., 1998), we examined the possibility whether *Nf2* inactivation altered signal transduction important for progenitor proliferation and differentiation. As expected, we detected intense staining for phospho-tyrosine (p-tyrosine), commonly found in activated receptor tyrosine kinases (RTKs), in the apical surface of CTL neural tubes at E11.5 (Fig. 7A). Also, stronger p-tyrosine labeling was observed in the neuronal layer. A similar intense p-tyrosine staining pattern was found in the apical surface of CTL neural tubes at E8.5 and E9.5 (Supplementary Fig. S5A,B). Consistent with p-tyrosine staining, phospho-epidermal growth factor receptor (p-EGFR) was enriched on the apical surface of CTL neural tubes (Fig. 7C). In addition, we detected strong cytoplasmic and nuclear staining of phospho-AKT (p-AKT) (Fig. 7E) and phospho-ERKs (p-ERKs) (not shown), which are downstream signaling molecules of RTKs, in CTL neural tubes at E11.5 and earlier embryonic time points E8.5 and E9.5 (Supplementary Fig. S5C,D). In contrast, very weak p-tyrosine and p-EGFR staining was found throughout the entire *Nf2*$^{Nestin}$CKO neuroepithelium (Fig. 7B,D), and p-AKT staining was barely detectable (Fig. 7F). Together, these results contrast with the finding of *Nf2* deficiency-induced up-regulation of EGFR signaling in cultured embryonic fibroblasts (Curto et al., 2007) and suggest that *Nf2* inactivation in the neuroepithelium may inhibit progenitor cell proliferation.

To test this possibility, we stained embryo sections for Cyclin $D_1$, a G1-cyclin important for

$G_1$-to-S progression. Intense nuclear labeling of Cyclin $D_1$ was detected in the majority of neuroprogenitor cells in CTL neural tubes at E11.5 (Fig. 7G) and earlier embryonic time points E8.5 and E9.5 (Supplementary Fig. S5E,F). In contrast, very few cells in the VZ of the *Nf2*$^{Nestin}$CKO neuroepithelium expressed Cyclin $D_1$, and the Cyclin $D_1$-positive cells were dispersed towards the basal side of the neuroepithelium (Fig. 7H). To corroborate this finding, we examined the expression of Ki67, a proliferating cell marker present in all active phases of the cell cycle. As expected, neuroprogenitor cells in the entire VZ of CTL neural tubes at E11.5 (Fig. 7I) and earlier embryonic time points E8.5 and E9.5 (Supplementary Fig. S5G,H) were labeled with Ki67. In contrast, the thickness of the VZ in *Nf2*$^{Nestin}$CKO neural tubes was greatly reduced, and a few Ki67+ cells were dispersed outside the VZ (Fig. 7J). Furthermore, immunostaining for pH3, which labels mitotic cells, detected very few pH3-positive cells in the mutant neural tube, in contrast to a contiguous layer of pH3-positive cells in the VZ near the apical surface of the CTL neural tube (Fig. 7K,L). Importantly, the reduction of pH3-positive cells in the apical neuroepithelium was also observed in *Nf2*$^{Wnt1}$CKO embryos (Fig. 2D-F). Collectively, these results from two independent gene-targeting studies strongly suggest that *Nf2* inactivation results in depletion of the neuroprogenitor pool in the developing neural tube.

*Nf2 inactivation in neuroprogenitor cells during mid-to-late gestation led to schwannoma formation.* Tamoxifen-induced *Nf2* inactivation in *Nestin-CreER;Nf2*$^{flox2/flox2}$ mice prior to E12.5 did not yield any live pups, likely due to defects in neural tube development and other manifestations noted above (Fig. 4A). To examine whether *Nf2* inactivation at later stages of embryonic development could lead to tumorigenesis, we injected tamoxifen into pregnant *Nestin-CreER;Nf2*$^{flox2/flox2}$ mice from 12.5 to 16.5 days p.c. We obtained one or two live pups

from pregnant dams injected with tamoxifen at 12.5 or 13.5 days p.c., respectively, and about six pups when tamoxifen was administered at 14.5 days p.c. More pups were born when tamoxifen was administered at later time points. We obtained an average of eight pups per litter when tamoxifen was injected at 15.5 ~16.5 days p.c. (Supplementary Fig. S6). All of the mice were monitored for tumor formation and other abnormalities during their lifespan (up to two years). Interestingly, about 52% (17/33) of these mice developed tumors [schwannomas (6/17), lymphomas (2/17), or both (9/17)] by 18 months of age with the earliest detection at about 7 months. In several instances, multiple schwannomas were found in the same mouse (Fig. 8A). Schwannomas developed in these *Nf2*$^{Nestin}$CKO mice were encapsulated, globoid, whitish masses that occurred in the proximal extremities, the facial or neck area, or the retroperitoneum. Microscopically, the tumors consisted of spindle-shaped cells, and some areas showed occasional nuclear palisading (Antoni A pattern) adjacent to less cellular Antoni B areas (Fig. 8B). Also, the tumor cells displayed immunoreactivity to Schwann cell markers S100 and MBP (Fig. 8E,F). All of these characteristics are consistent with a primary schwannoma. About 12% of schwannoma cells in a given section stained positive for Ki67 (Fig. 8G), and some of the tumors had necrosis in the center (not shown). In addition to schwannomas, some *Nf2*$^{Nestin}$CKO mice also developed an abnormally large thymus (Fig. 8C) filled with lymphoma cells with lymphoblastic characteristics (Fig. 8D). Some micrometastases of lymphoma cells were found in the liver, kidney, and lung. Importantly, the tumor cells were negative for merlin immunostaining (Fig. 8H-J). These results suggest that biallelic loss of the *Nf2* gene in neuroprogenitor cells during mid-to-late gestation could result in tumor formation, including schwannomas.

*Both $Nf2^{Nestin}$*CKO *mouse schwannoma and human vestibular schwannoma cells displayed activated β–catenin, AKT, and MAPK signaling pathways.* To examine whether merlin-deficient schwannoma cells exhibit altered expression patterns for AJ components, we stained sections of normal sciatic nerves isolated from CTL ($Nf2^{flox2/flox2}$) mice and schwannomas from $Nf2^{Nestin}$CKO mice for β-catenin, E-cadherin, and N-cadherin. It has been shown that N-cadherin is concentrated in the node of Ranvier in the sciatic nerve (Cifuentes-Diaz et al., 1994), while both β-catenin and E-cadherin are enriched at the paranodal region (Fannon et al., 1995). We also detected β-catenin in the cytoplasm and membrane of Schwann cells in normal sciatic nerves. Intense β-catenin staining was seen in specific membrane locations reminiscent of the paranodal regions (Fig. 9A). Similarly, we found E-cadherin mainly in specific membrane locations, like the paranodal regions. In addition, we observed robust N-cadherin expression in the cytoplasm and membrane of normal Schwann cells, and intense N-cadherin staining appeared as a thin line at the node of Ranvier. In contrast, strong β-catenin expression was detected in the cytoplasm of schwannoma cells from $Nf2^{Nestin}$CKO mice and some staining appeared in the nucleus (Fig. 9B). Intriguingly, we did not find E-cadherin in $Nf2^{Nestin}$CKO schwannoma cells, while we detected intense N-cadherin staining in the cytoplasm, particularly in the perinuclear region.

Since $Nf2^{Nestin}$CKO schwannoma cells expressed abundant β-catenin, we examined the possibility of activation of β-catenin's downstream signals in the nucleus. Interestingly, we readily detected both Tcf1 and cyclin $D_1$ in the nuclei of these mouse schwannoma cells (Fig. 9B), whereas Tcf1 and cyclin $D_1$ were not found in normal Schwann cells (Fig. 9A), indicating that β-catenin's downstream signals are activated in $Nf2^{Nestin}$CKO schwannomas but not in normal Schwann cells. To validate these findings in humans, we performed similar immunostainings on human vestibular schwannoma tumor sections. As in $Nf2^{Nestin}$CKO mouse

schwannomas, human vestibular schwannoma cells expressed high levels of β-catenin, mostly found in the cytoplasm (Fig. 9C). In addition to nuclear Tcf1, these human tumor cells express β-catenin's downstream signals c-Myc and cyclin $D_1$ in the nucleus and cytoplasm. Curiously, both *Nf2*$^{Nestin}$CKO schwannoma cells and normal Schwann cells expressed GSK-3β (Fig. 9A,B), a negative regulator of the Wnt/β-catenin signaling pathway (Klaus and Birchmeier, 2008). Nevertheless, these results suggest that the β-catenin pathway is activated in both *Nf2*$^{Nestin}$CKO mouse schwannoma and human vestibular schwannoma cells.

In addition to regulating cell:cell contact, merlin has been shown to coordinate receptor signaling (Curto et al., 2007; Lallemand et al., 2009). Consistent with these findings, we detected high levels of p-tyrosine accompanied by strong activation of the downstream targets of several RTKs, including ERKs, AKT, and JNK, in *Nf2*$^{Nestin}$CKO schwannomas (Fig. 10A). Conversely, we found only very low levels of p-tyrosine, p-ERKs, p-AKT, and p-JNK in normal Schwann cells (Fig. 10B). To further examine expression of other key components of the PI3K/AKT pathway, we stained tissue sections for PI3K, PTEN, and p-PTEN. While some PTEN expression was detected, strong p-PTEN staining was found in the nucleus and cytoplasm of *Nf2*$^{Nestin}$CKO schwannoma cells (Fig. 10A), suggesting that the majority of PTEN is phosphorylated. Consistent with AKT activation, high levels of PI3K were detected in the nucleus and cytoplasm of these mouse tumor cells. However, only trace amounts of p-AKT was seen in the cytoplasm of normal Schwann cells (Fig. 10B). Thus, as in human vestibular schwannomas (Tang et al., 2007; Jacob et al., 2008; Lallemand et al., 2009; Lee et al., 2009), the PI3K/AKT pathway is activated in *Nf2*$^{Nestin}$CKO mouse schwannomas. In addition, we detected robust staining of p-JNK and p-c-Jun in the nucleus and cytoplasm of *Nf2*$^{Nestin}$CKO schwannomas, indicating activation of the JNK pathway (Fig. 10A). Together, these results

imply that multiple signaling pathways, including the β–catenin, AKT, and MAPK pathways, are activated in both *Nf2*$^{Nestin}$CKO mouse schwannomas and human vestibular schwannomas.

## DISCUSSION

Studies indicate that tumor suppressor genes often play important roles during embryonic development and the outcome of tumor suppressor gene inactivation is time- and cell type-dependent (Weinberg, 1993; Lee et al., 1995; Vogelstein and Kinzler, 2004; Cully et al., 2006; Klaus and Birchmeier, 2008). For example, mutations in the *NF2* gene are frequently associated with a subset of nervous system tumors, including vestibular schwannomas (reviewed in Welling et al., 2008). However, the timing of biallelic *NF2* loss resulting in this cell type-specific tumorigenesis remains to be examined. Although *Nf2* function is essential during early embryonic development (McClatchey et al., 1997) and is important for tissue fusion, including neural tube closure (McLaughlin et al., 1997), the mechanism by which *Nf2* loss causes NTDs and whether merlin plays any roles at later stages of development are not understood. In this paper, we show that merlin is important for neural tube closure and neural crest cell formation and migration. Using the tamoxifen-inducible *Nestin-CreER* driver, we further demonstrate that *Nf2* inactivation in neuroprogenitor cells during early gestation disrupts neuroepithelial cell integrity, abolishes the formation of apical AJs and associated RTK signaling, depletes the neuroprogenitor pool, and affects neuronal differentiation, consequently leading to NTDs. In contrast, *Nf2* inactivation during late gestation results in the formation of schwannomas and/or lymphomas at a high frequency. Interestingly, like human vestibular schwannomas, these *Nf2*$^{Nestin}$CKO mouse schwannomas exhibit activation of multiple signaling pathways, including the AKT, MAPK, and β-catenin pathways. Our results indicate that merlin is essential for

neurulation and neuroprogenitor proliferation, and further identify an unprecedented role for merlin in promoting cell proliferation during mammalian brain development, which contrasts with its function as a tumor suppressor in specific cell types, such as Schwann cells (Fig. 11).

Neural tube closure involves cell adhesion, migration, proliferation and apoptosis. Although many genes have been implicated in this important developmental process (Harris and Juriloff, 2007), the cellular mechanisms underlying neural tube closure are not well-defined. By inactivating *Nf2* in the dorsal neural tube or in neuroprogenitor cells during early gestation, we demonstrated that merlin function is essential during neural tube closure, NCC migration, and neuroprogenitor cell proliferation. These results coincide with a role for merlin in tissue fusion (McLaughlin et al., 2007) and further imply that the dynamic change in *NF2* expression during neural tube closure and NCC migration may be a means to regulate merlin function during these processes (Akhmametyeva et al., 2006). Consistent with a role for merlin in regulating cadherin-mediated cell:cell contact (Lallemand et al., 2003), we found that neuroepithelia in $Nf2^{Nestin}$CKO and $Nf2^{Wnt1}$CKO embryos were poorly organized and exhibited cell detachment. In the absence of merlin, β-catenin and N-cadherin could not be targeted to the membrane; however, it is not known why these AJ components disappeared in the mutant neuroepithelial cells. Since merlin coordinates endocytic trafficking in epithelial cells (Curto et al., 2007; Lallemand et al., 2009), we speculate that N-cadherin and β-catenin may be targeted for degradation in merlin-deficient neuroepithelial cells. This hypothesis warrants further investigation.

In addition to its roles in the maintenance of tissue integrity and epithelial cell polarity (Delva and Kowalczyk, 2009), AJs can bind to growth factor receptors and allow concentrating signaling receptors and their target proteins to facilitate efficient transmission of signals between epithelial cells (Woods and Bryant, 1993; Chenn et al., 1998). Due to the lack of AJs in the

apical surface, merlin-deficient neuroepithelial cells lose their integrity and polarity and have very few p-tyrosine-containing RTKs. Without RTKs in specific membrane locations, neuroepithelial cells could not respond to growth-promoting signals from the apical surface and the signals mediated by cell-cell interactions for proliferation and differentiation. These findings implicate that merlin has pro-proliferation functions during early brain development. Also, our observation that the *Nf2*$^{Nestin}$CKO neuroepithelium contained an increased number of apoptotic cells is consistent with the notion of abnormal cell-cell adhesion and detachment-induced apoptosis in merlin-deficient cells during tissue fusion (McLaughlin et al., 2007).

It should also be mentioned that proper cadherin expression is critical for the formation of NCCs, which arise from the crest region of the developing neural tube. NCCs undergo an epithelial-to-mesenchymal transition and migrate to precise destinations in the embryo, where they differentiate into the peripheral nervous system, melanocytes, and the craniofacial structures (LeDouarin and Kalcheim, 1999). Our neural tube explant experiments highlight the important role of merlin in NCC adhesion, migration, and proliferation. Together with the short axonal extension, the absence of the ventral commissure, and the absence or delayed formation of cranial and spinal ganglia in *Nf2*$^{Wnt1}$CKO and *Nf2*$^{Nestin}$CKO embryos, these results further support the idea that merlin function is required for the development of the NCC-derived peripheral nervous system. In addition to NTDs, *Nf2*$^{Nestin}$CKO and *Nf2*$^{Wnt1}$CKO embryos displayed defects in the development of the branchial arches and heart. Experiments are in progress to examine the roles of merlin during the development of these organs. In addition, the observation that pregnant *Nestin-CreER;Nf2*$^{flox2/flox2}$ mice with tamoxifen treatment during mid-to late gestation have a low birth rate implies that merlin plays some role during later stages of neural development.

In contrast to pro-proliferative roles during early brain development, merlin possesses growth suppression functions (reviewed in McClatchey and Giovannini, 2005). In cultured cells, over-expression of the *Nf2* gene can limit cell growth, and mouse embryo fibroblasts lacking merlin do not undergo contact-dependent growth arrest (Lallemand et al., 2003). *Nf2* inactivation in mice causes tumor development and may contribute to tumor metastasis (McClatchey et al., 1998; Giovannini et al., 2000; Kalamarides et al., 2002; Morris and McClatchey, 2009). In addition, inactivating mutations and loss of heterozygosity in the *NF2* gene are frequently found in NF2-associated tumors, such as vestibular schwannomas (Welling et al., 2008). Consistent with these findings, we found that $Nf2^{Nestin}$CKO mice with *Nf2* inactivation during mid-to-late gestation frequently developed schwannomas and lymphomas. It should be noted that $Nf2^{Nestin}$CKO mice are in a FVB/N:C57BL/6 mixed background and we do not known whether the genetic background could affect the incidence and spectrum of tumor formation. The development of lymphomas in $Nf2^{Nestin}$CKO mice is consistent with the notion that Nestin represents a characteristic marker of multi-lineage progenitor cells (Zimmerman et al., 1994; Wiese et al., 2004) and further suggests that the enhancer in the 2$^{nd}$ intron of the *Nestin* gene in the *Nestin-CreER* construct can direct transgene expression to both neural and non-neural progenitor cells. Curiously, lymphomas were also frequently observed in *Nf2*–knockout mice (McClatchey et al., 1998). In addition, both schwannomas and lymphomas have been found in patients with NF2 (Dohi et al., 2006), and *NF2* mutations have been found in acute lymphoblastic lymphomas (Kawamata et al., 2009).

E-cadherin and N-cadherin have long been implicated in tumor progression and metastasis. Loss of E-cadherin expression or mutations in the E-cadherin gene have been detected in a variety of epithelial cancers and metastases (Jeanes et al., 2008). We also found loss of E-

cadherin expression in *Nf2*$^{Nestin}$CKO schwannomas; however, it is not known whether loss of E-cadherin promotes tumorigenesis. Conversely, aberrant expression of N-cadherin along with the loss of E-cadherin function has been detected in human cancers. This cadherin switching may play an important role in the behavior of tumor cells, as over-expression of N-cadherin can promote cell motility and survival (Wheelock et al., 2008). Importantly, unlike those neuroprogenitor cells with *Nf2* loss during early gestation, which had little N-cadherin and β-catenin, *Nf2*$^{Nestin}$CKO schwannoma cells expressed high levels of N-cadherin in the cytoplasm but not in the membrane. Also, these schwannoma cells displayed strong β-catenin staining in the cytoplasm. These results suggest that *Nf2*$^{Nestin}$CKO schwannoma cells have acquired a mechanism to stabilize β-catenin and N-cadherin in the absence of merlin. Since GSK-3β, which phosphorylates β-catenin and targets it to degradation, is a downstream substrate of PI3K/AKT (Klaus and Birchmeier, 2008), it is possible that activated AKT may stabilize β-catenin by phosphorylating and inactivating GSK-3β in schwannoma cells (Sharma et al., 2002). Alternatively, activated AKT may directly phosphorylate β-catenin at its C-terminal region and promote its nuclear translocation (He et al., 2007). In addition, loss of E-cadherin expression may contribute to up-regulation of β-catenin signaling, and changes in the cellular location of N-cadherin may cause redistribution of the β-catenin pool, thereby influencing the expression of β-catenin target genes (Gottardi et al., 2001). The detection of β-catenin's binding partner Tcf1 and its downstream targets cyclin $D_1$ and c-Myc in the nucleus of *Nf2*$^{Nestin}$CKO mouse schwannoma and human vestibular schwannoma cells implies that activation of the β-catenin pathway may be a common mechanism to promote schwannoma cell growth. In addition, like human vestibular schwannomas (Tang et al., 2007; Jacob et al., 2008; Lallemand et al., 2009; Lee et al., 2009), *Nf2*$^{Nestin}$CKO mouse schwannomas display activation of multiple signaling pathways.

Experiments are in progress to investigate at what stages these signaling pathways are activated in *Nf2*$^{Nestin}$CKO mice. Also, we are conducting experiments to examine whether *Nf2* inactivation during mid-to-late gestation affects neuroprogenitor cell proliferation and/or differentiation, whether *Nf2* inactivation after birth can lead to tumor formation, and whether *Nf2* inactivation at different time points affects the spectrum of tumor formation and their growth characteristics.

In summary, our results argue that merlin may function to inhibit or promote cell growth dependent on the biological context. Our findings further imply that mutations that inactivate the 2$^{nd}$ allele of the *NF2* gene found in NF2-associated tumors most likely occur after neural tube development is completed since *Nf2* inactivation during early gestation results in embryonic lethality due to NTDs or other manifestations. The relatively high frequency of tumor formation in *Nf2*$^{Nestin}$CKO mice suggests that they may be used as an alternative model for NF2-associated schwannomas.

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**Figure Legends**

Fig. 1. Merlin function is required for neural tube closure. (A) PCR analysis showing considerable deletion of the floxed *Nf2* allele in the midbrain of an E8.5 $Nf2^{Wnt1}$CKO embryo. Yolk sacs (left image) or midbrains (right image) were used to genotype the wild-type (WT) and floxed *Nf2* alleles, or the WT and deleted alleles in the presence of Cre according to Giovannini et al. (2000). Lanes 1-3 are controls for the $Nf2^{WT/WT}$, $Nf2^{WT/flox2}$, and $Nf2^{flox2/flox2}$ genotypes, respectively. Lane 4 contains samples from $Nf2^{Wnt1}$CKO embryos. M, molecular-weight marker of 1-kb Plus DNA Ladder (Invitrogen). (B) $Nf2^{Wnt1}$CKO embryos at E8.5 ~ E9.0 displayed defects in neural tube closure (arrow). CTL, control; MB, midbrain; HB, hindbrain. Allantois is indicated with an asterisk.

Fig. 2. *Nf2* inactivation in the dorsal neural tube causes the disappearance of F-actin structures, impairs the mitotic activity in the VZ, and affects neuronal differentiation. Frozen transverse sections of E9.5 $Nf2^{Wnt1}$CKO (C,F,I) and E8.5 (A,D,G) and E9.5 (B,E,H) CTL embryos were stained with phalloidin for F-actin (A,B,C) or with antibodies against pH3 (D,E,F) or β-tubulin III (TuJ1; G,H,I). DAPI stained nuclei blue. Arrows point to the apical surface or the unclosed neural tube (asterisk). The ventral commissure, the structure where axons cross from one side to the other of the ventral midline of the neural tube, is marked by a filled arrowhead and was not found in the CKO neural tube. Also, cranial ganglia were only found in the CTL embryo (open arrowheads).

Fig. 3. *Nf2* inactivation abolished NCC adhesion and migration from $Nf2^{Wnt1}$*CKO* neural tube explants. Dorsal neural tubes (NT) from E9.5 wild-type (CTL) and $Nf2^{Wnt1}$*CKO* embryos were dissected and cultured in fibronectin-coated dishes for six days as described in Methods. In contrast to CTL cultures (A,B), the $Nf2^{Wnt1}$*CKO* neural tube explants could not adhere to the

substratum (C) or exhibited weak adhesion to the substratum but were unable to proliferate or initiate migration (D). To examine the effect of merlin knockdown, cultures of E9.5 CTL neural tube explants were either mock-infected (E,F) or infected with a lentiviral vector expressing *Nf2* shRNA lentiviral particles (G,H). Note that neural tube explant cultures with *Nf2* shRNA knockdown displayed less migrating epithelial cells on the bottom layer but had numerous glial extensions forming spider web-like structures as compared to mock-infected cultures.

Fig. 4. *Nf2* inactivation in neuroprogenitor cells using the inducible *Nestin-CreER* line also causes NTDs. Tamoxifen was injected into pregnant *Nestin-CreER;Nf2*$^{flox2/flox2}$ mice at E7.5 ~ E8.5, and embryos were harvested at E10.5 ~ E11.5 for analysis. (A) *Nf2*$^{Nestin}$CKO embryos displayed NTDs, including exencephaly (arrowhead). CTL, control. (B-C) Hematoxylin-eosin staining of transverse (B) and sagittal (C) paraffin sections of CTL and *Nf2*$^{Nestin}$CKO embryos showing the poorly-developed neural tube in CKO embryos. (D-E) In contrast to a tightly-organized neuroepithelium in the CTL embryo (D), the unclosed neural tubes in the CKO embryo contained fewer, loosely-organized cells (E). Images shown correspond to the neural tube regions denoted by the rectangle in (C). Ap, apical surface; B, basal lamina. (F-G) Images of double immunostaining of the CTL (F) or CKO (G) neural tubes for neuronal β-tubulin III (TuJ1) and cleaved caspase 3 (CC3). DAPI stained nuclei blue. (H-K) Efficiency of *Nestin-CreER*-mediated *Nf2* deletion in neuroprogenitor cells. PCR analysis demonstrating *Nestin-CreER*-mediated recombination in *Nf2*$^{Nestin}$CKO embryos (H). Lanes 1-2 are controls for the deleted (lane 1) and floxed (lane 2) *Nf2* alleles, respectively. Lanes 3-4 contain brain samples from two *Nf2*$^{Nestin}$CKO embryos. M, molecular-weight markers. Images of immunostaining of the CTL (J) and CKO (I,K) neural tubes for Cre (I) or merlin (J,K). Arrows point to the apical surface (Ap).

Fig. 5. *Nf2* inactivation disrupts the formation of apical AJs. Sagittal paraffin sections of E11.5 control (CTL) (A,C) and *Nf2*$^{Nestin}$CKO (B,D) embryos were immunostained for N-cadherin (A,B) and β-catenin (C,D). DAPI stained nuclei blue. Note that the N-cadherin- and β-catenin-containing AJs on the apical surface (Ap) were greatly diminished in the CKO neuroepithelium.

Fig. 6. Altered JNK signaling in the *Nf2*$^{Nestin}$CKO neuroepithelium. Sagittal sections of CTL (A,B,C) and *Nf2*$^{Nestin}$CKO (D,E,F) neural tubes were doubly stained for p-JNK (A,D) and TuJ1 (B,E). Merged images were also shown (C,F). Ap, apical surface; B, basal lamina.

Fig. 7. Loss of merlin impairs apical AJs-associated signaling and causes depletion of the neuroprogenitor pool. Sagittal sections of E11.5 control (CTL) (A,C,E,G,I,K) and *Nf2*$^{Nestin}$CKO (B,D,F,H,J,L) embryos were stained for p-tyrosine (A,B), p-EGFR (C,D), p-AKT (E,F), cyclin $D_1$ (G,H), and Ki-67 (I,J). Hematoxylin was used as a counterstain. Also, sections were doubly immunostained for pH3 and MAP2 (K,L). DAPI stained nuclei blue. Note that very little immunoreactivity for p-tyrosine, p-EGFR, and p-AKT was detected in the *Nf2*$^{Nestin}$CKO neuroepithelium, consequently inhibiting neuroprogenitor cell proliferation. Ap, apical surface; B, basal lamina.

Fig. 8. *Nf2* inactivation in neuroprogenitor cells during late gestation results in tumorigenesis. Multiple schwannomas were found in a one-year-old *Nf2*$^{Nestin}$*CKO* mouse (A). Hematoxylin-eosin staining of a schwannoma section revealed spindle-shaped tumor cells with pleomorphic nuclei (B). A *Nf2*$^{Nestin}$CKO mouse exhibited an abnormally large thymus (C) filled with tumor cells with lymphoblastic characteristics (D). Schwannomas developed in the *Nf2*$^{Nestin}$*CKO* mouse expressed Schwann cell markers S100 (E) and MBP (F), and some of them were Ki67-positive (G). In contrast to the surrounding normal tissue, which expressed merlin

(green), tumor cells (asterisk) did not (H). DAPI stained nuclei blue (I). A merged image is shown (J).

Fig. 9. Expression of the β–catenin pathway in *Nf2$^{Nestin}$CKO* mouse schwannomas and human vestibular schwannomas. Sections of normal sciatic nerves (A) and *Nf2$^{Nestin}$CKO* schwannomas (B) were stained for β-catenin, E-cadherin, N-cadherin, Tcf1, cyclin $D_1$, and GSK-3β. Also, sections of a vestibular schwannoma were stained for β-catenin, Tcf1, cyclin $D_1$, and c-Myc (C). Note that while Schwann cells in normal sciatic nerves expressed β-catenin but lacked Tcf1 and cyclin $D_1$ staining, both *Nf2$^{Nestin}$CKO* mouse schwannoma and human vestibular schwannoma cells readily expressed β-catenin and its downstream signals.

Fig. 10. *Nf2$^{Nestin}$CKO* schwannoma cells displayed elevated levels of RTK-mediated downstream targets. (A) Sections of *Nf2$^{Nestin}$CKO* mouse schwannomas were stained for p-tyrosine, p-ERKs, p53, PI3K, PTEN, p-PTEN, p-AKT, p-c-Jun, and p-JNK. (B) Sections of normal sciatic nerves were stained for p-tyrosine, p-ERKs, p-AKT, and p-JNK. In contrast to normal sciatic nerves, *Nf2$^{Nestin}$CKO* schwannoma cells strongly expressed p-tyrosine, p-ERKs, PI3K, p-AKT, and p-JNK. However, little or no p53 staining was observed in *Nf2$^{Nestin}$CKO* schwannoma cells, indicating that the p53 gene is likely not mutated.

Fig. 11. Paradoxical roles of merlin during early brain development and tumorigenesis. In the developing neuroepithelium, merlin regulates the formation of cadherin-containing apical AJs, which play an important role in cell polarity and allow concentrating signaling receptors (RTKs) and their target proteins to facilitate efficient signal transduction. Both AJs and RTKs are important for neurulation and promote neuroepithelial progenitor proliferation. Conversely, merlin may act as a tumor suppressor in specific cell types, such as Schwann cells, to mediate contact-dependent inhibition of growth and metastasis. Merlin may regulate membrane levels of

multiple RTKs in these cells.

Figure 1

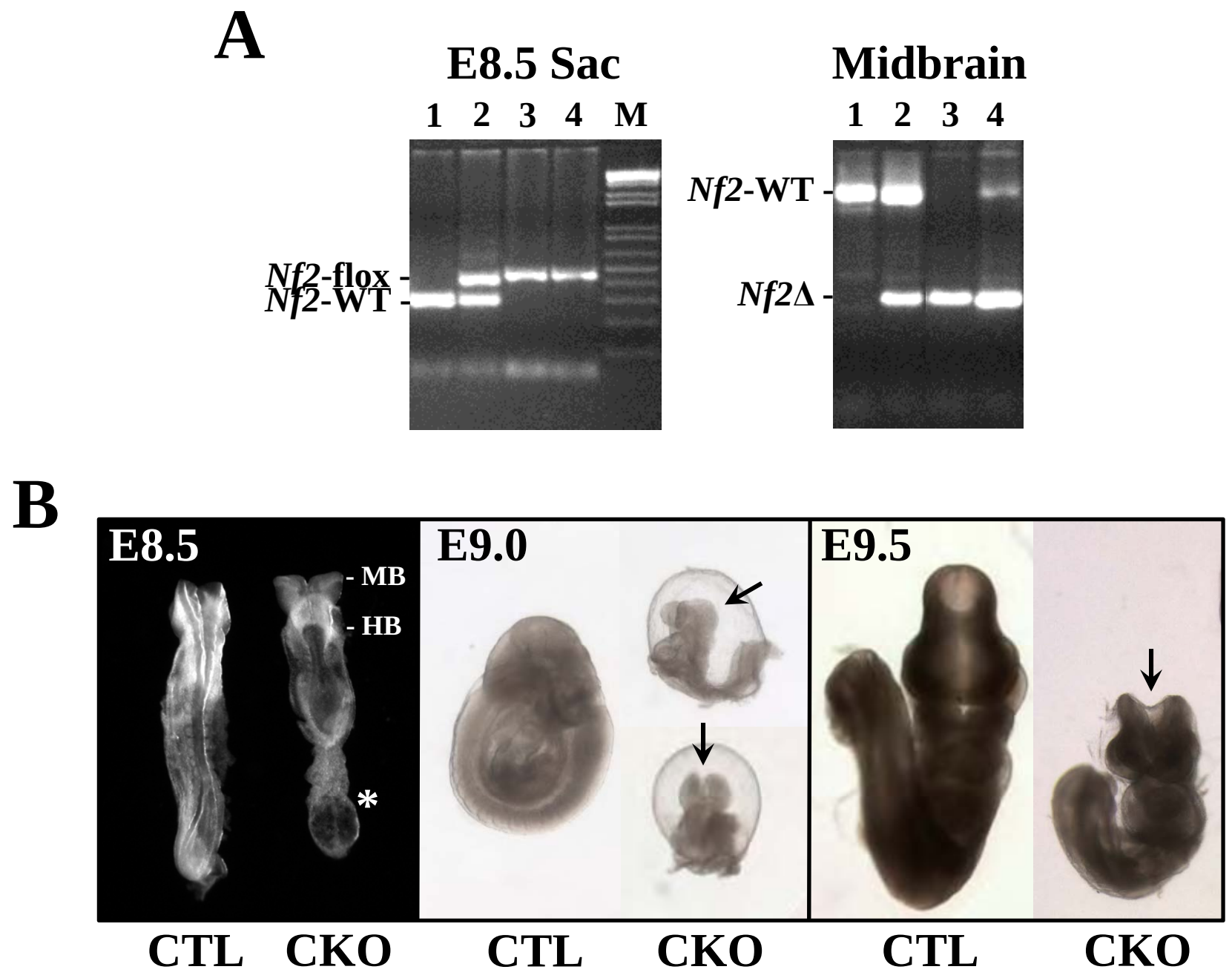
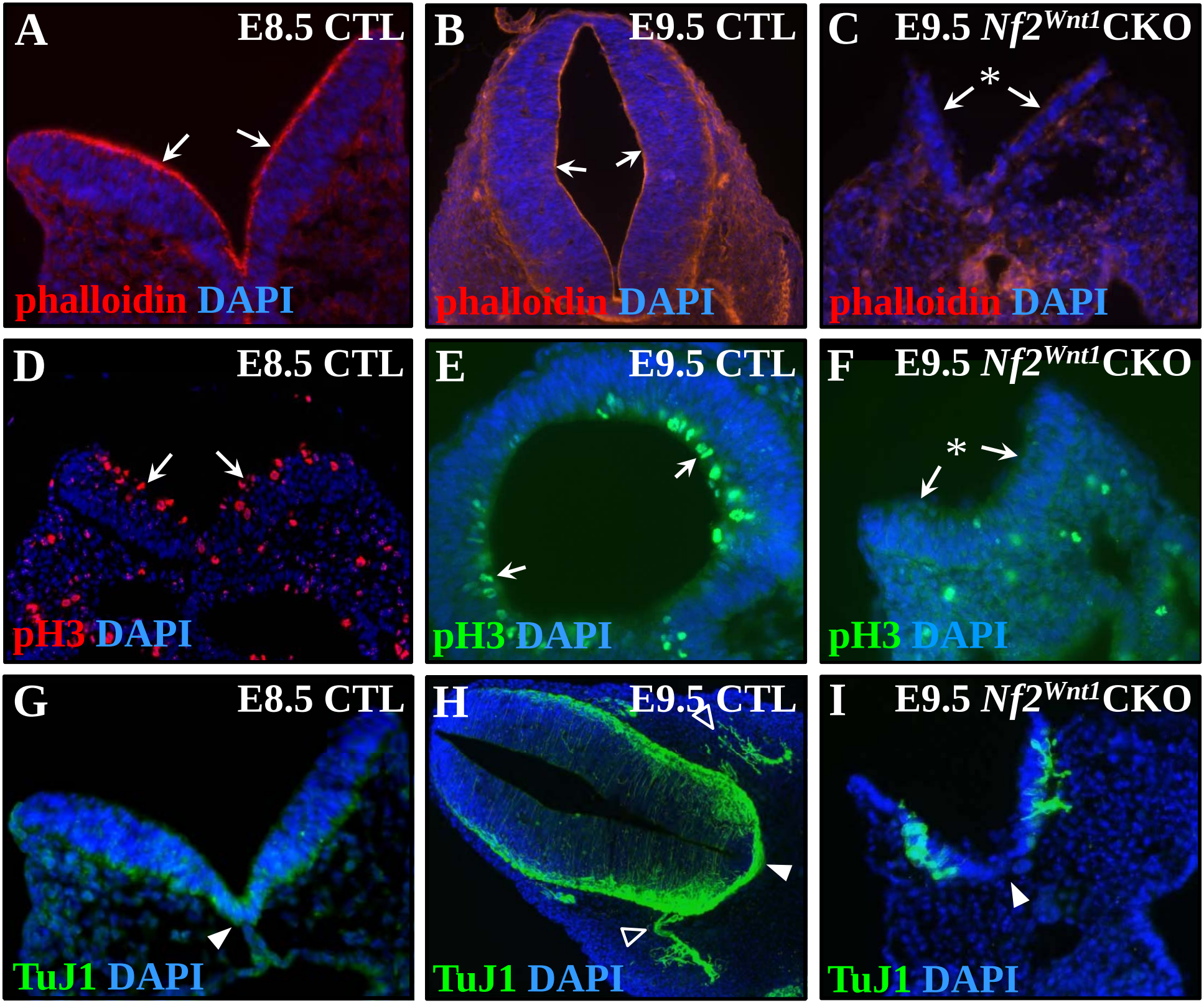

A
E8.5 Sac
1 2 3 4 M
Nf2-flox
Nf2-WT
Midbrain
1 2 3 4
Nf2-WT
Nf2Δ
B
E8.5
- MB
- HB
*
E9.0
E9.5
CTL CKO CTL CKO CTL CKO

Figure 2


A
E8.5 CTL
phalloidin DAPI
B
E9.5 CTL
phalloidin DAPI
C
E9.5 Nf2Wnt1CKO
phalloidin DAPI
D
E8.5 CTL
pH3 DAPI
E
E9.5 CTL
pH3 DAPI
F
E9.5 Nf2Wnt1CKO
pH3 DAPI
G
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TuJ1 DAPI
H
E9.5 CTL
TuJ1 DAPI
I
E9.5 Nf2Wnt1CKO
TuJ1 DAPI

Figure 3

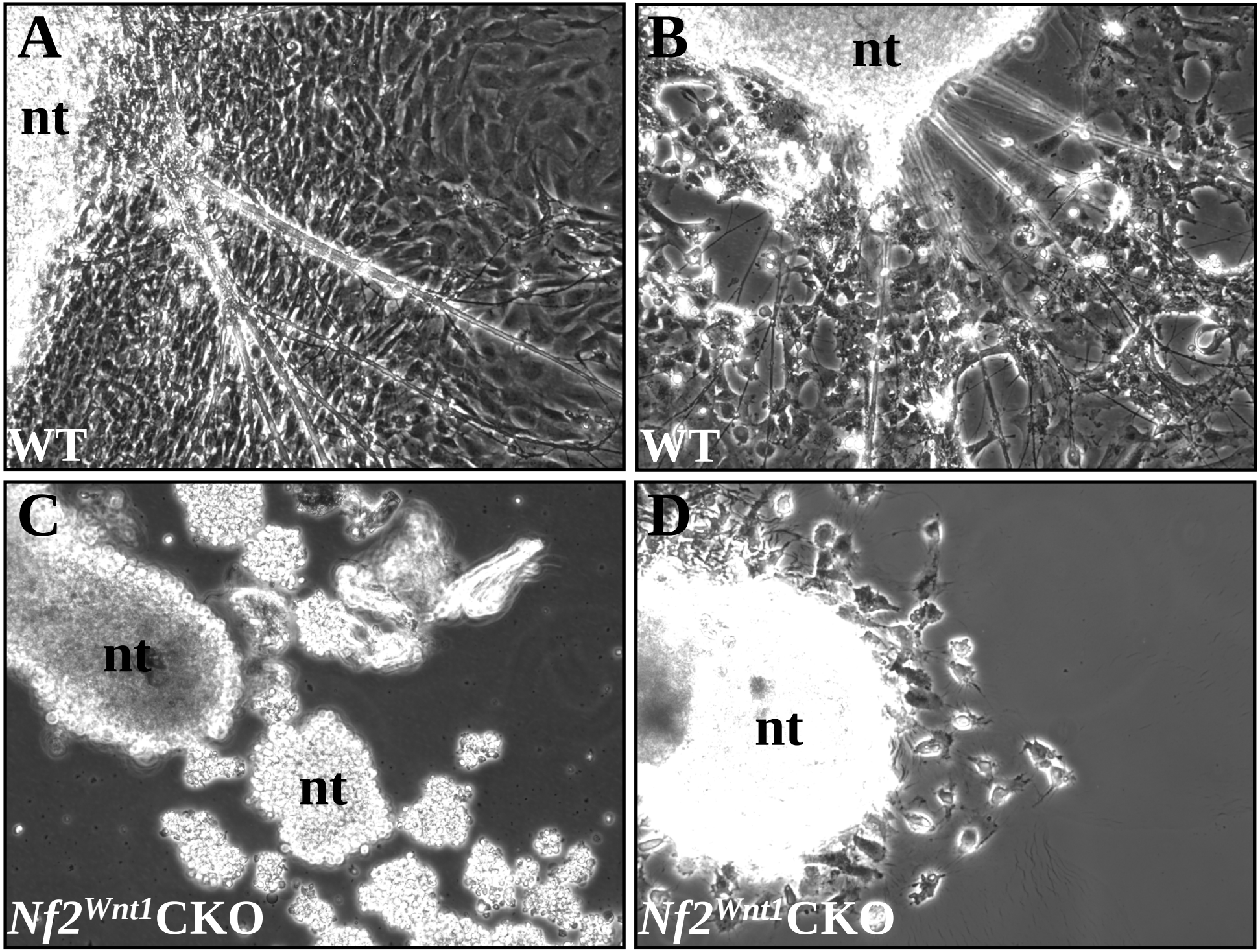


Figure 3 (Cont'd)

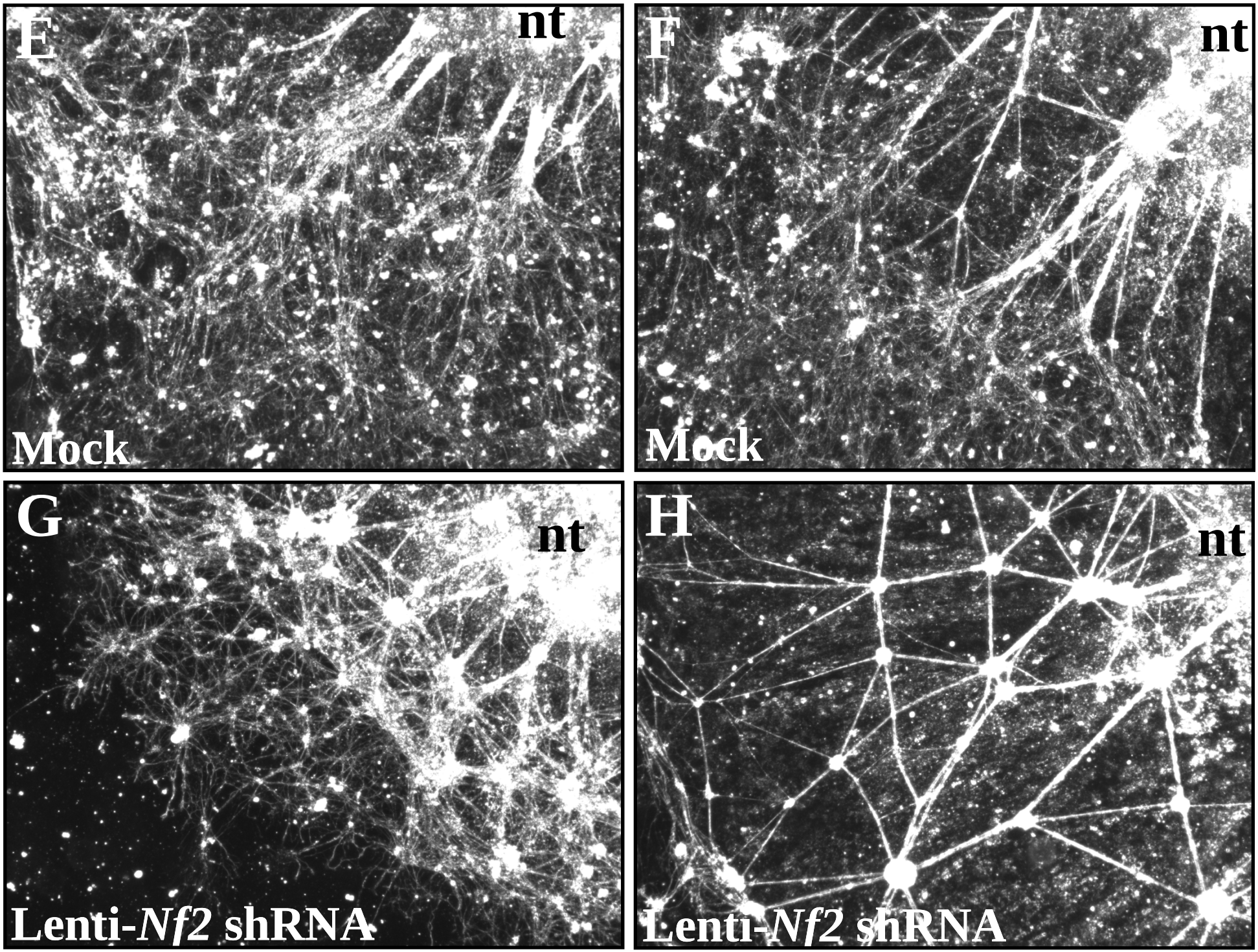


Figure 4

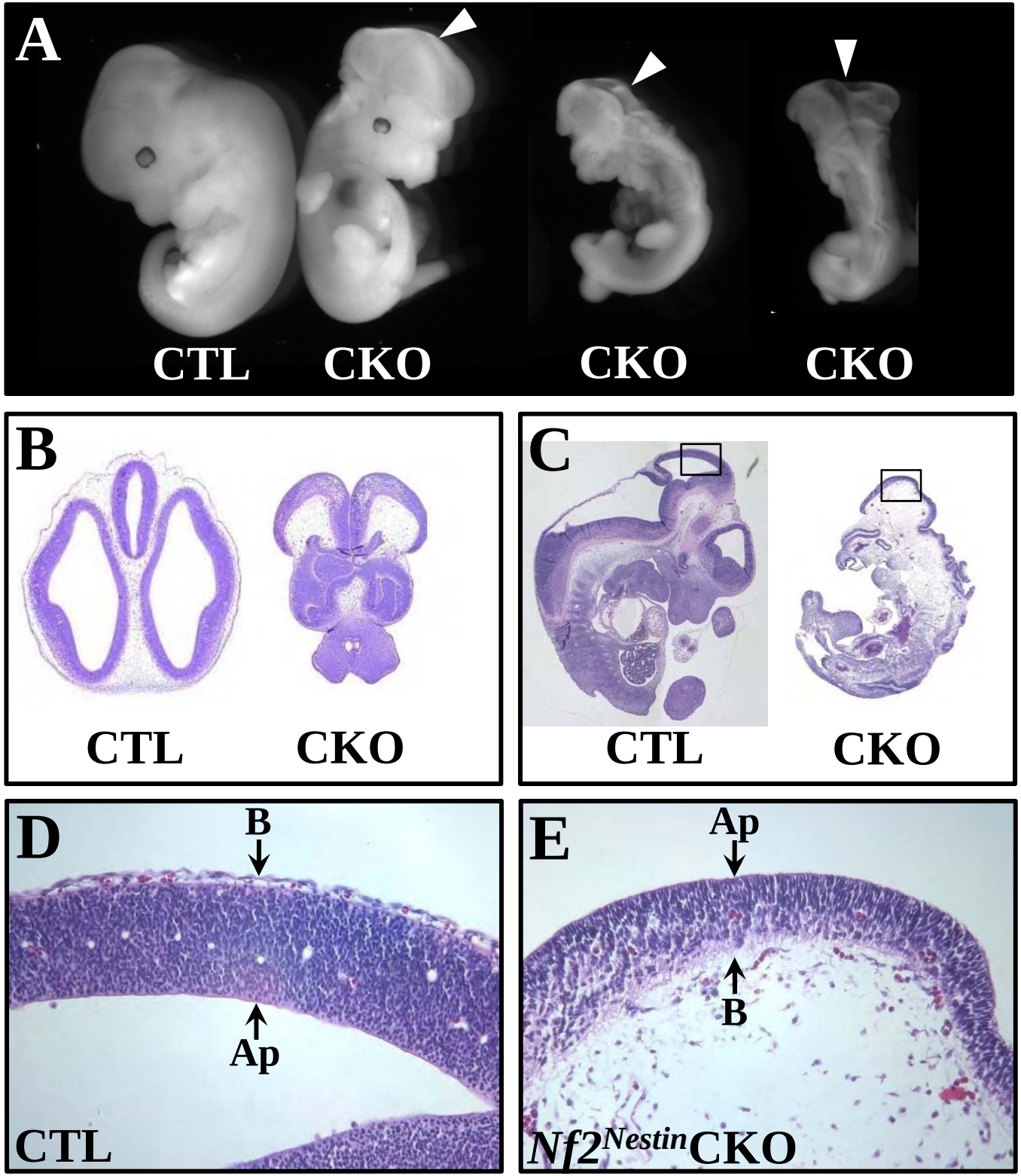

A
CTL
CKO
CKO
CKO
B
CTL
CKO
C
CTL
CKO
D
B
Ap
CTL
E
Ap
B
Nf2NestinCKO

Figure 4 (Cont'd)

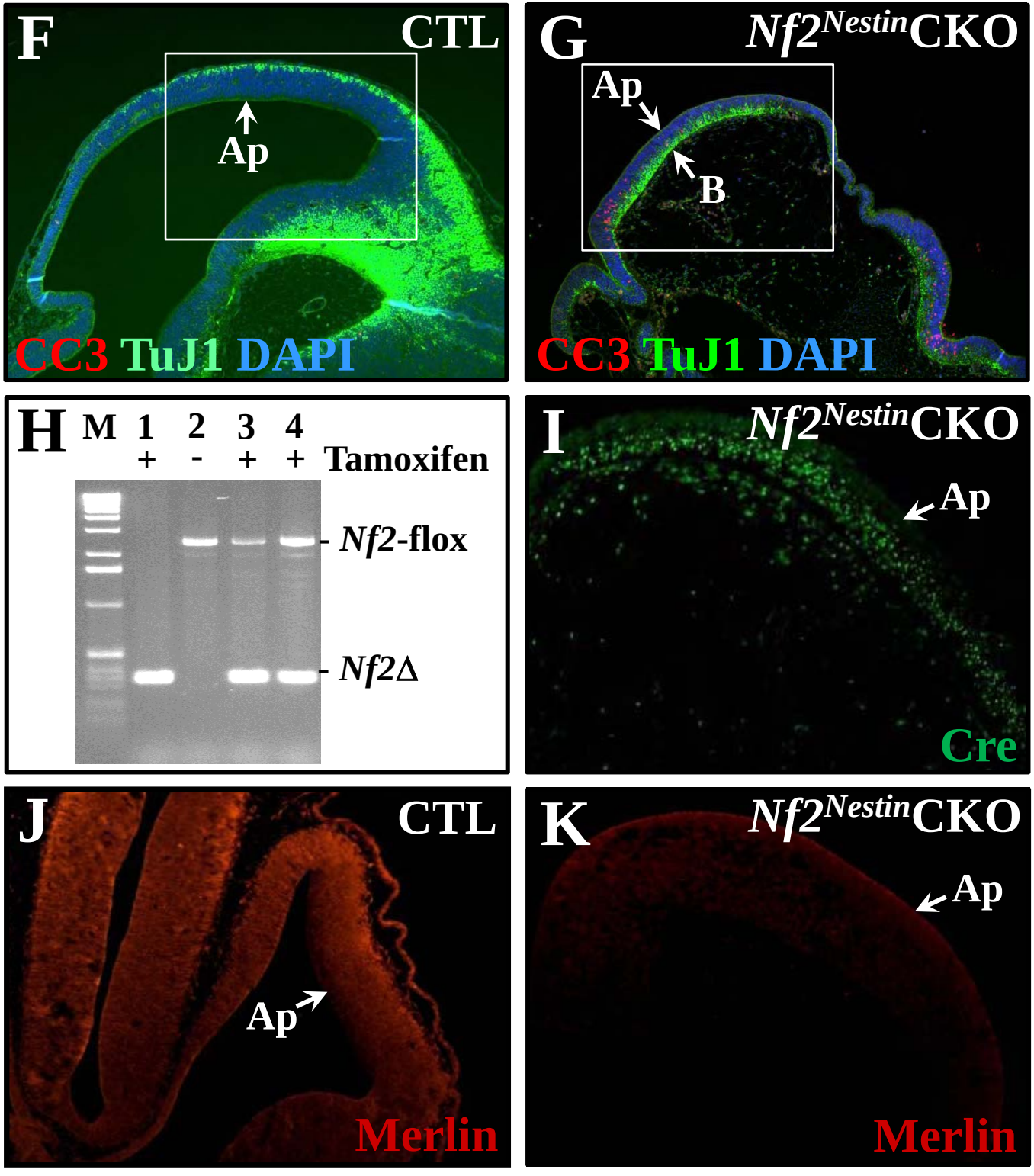


Figure 5

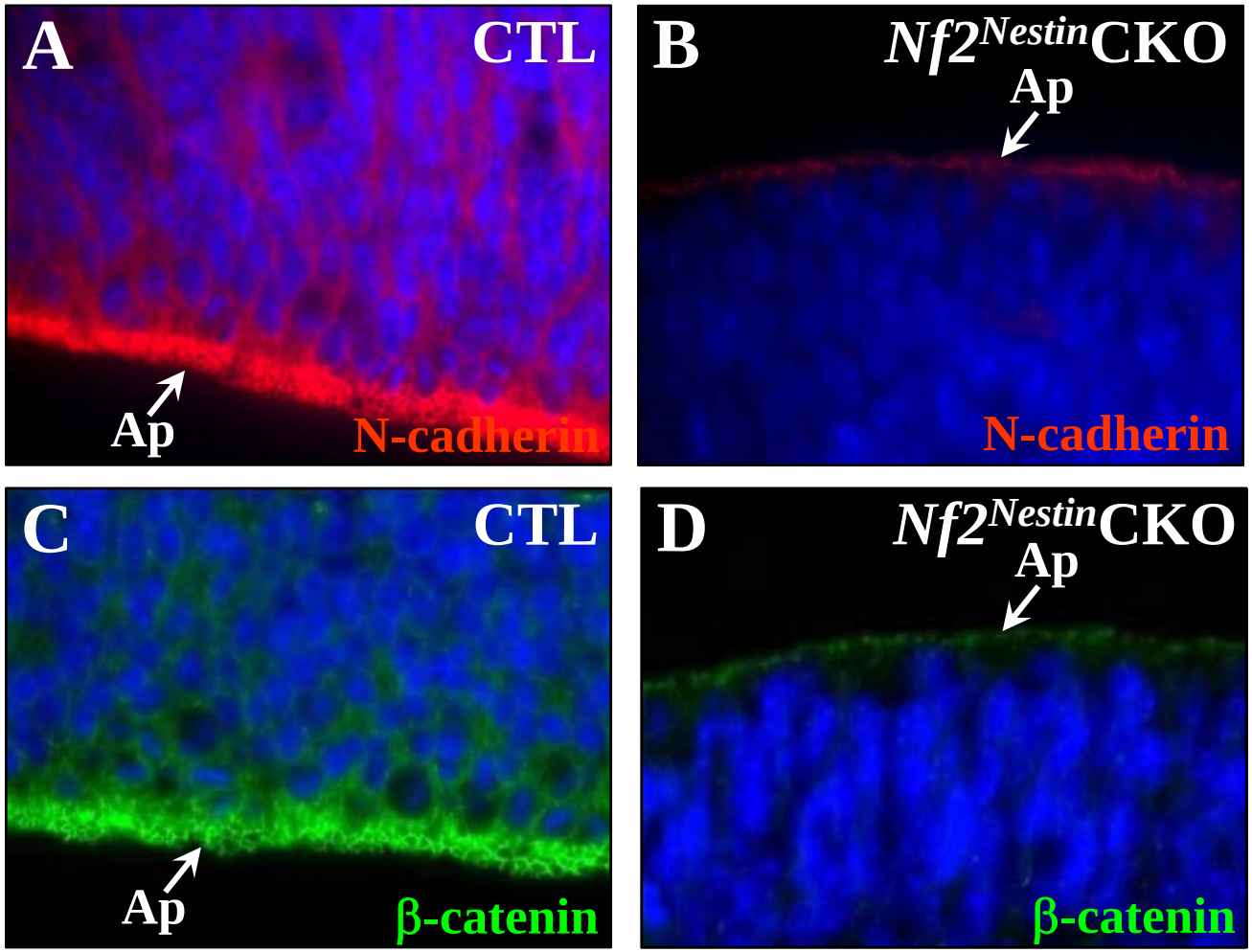
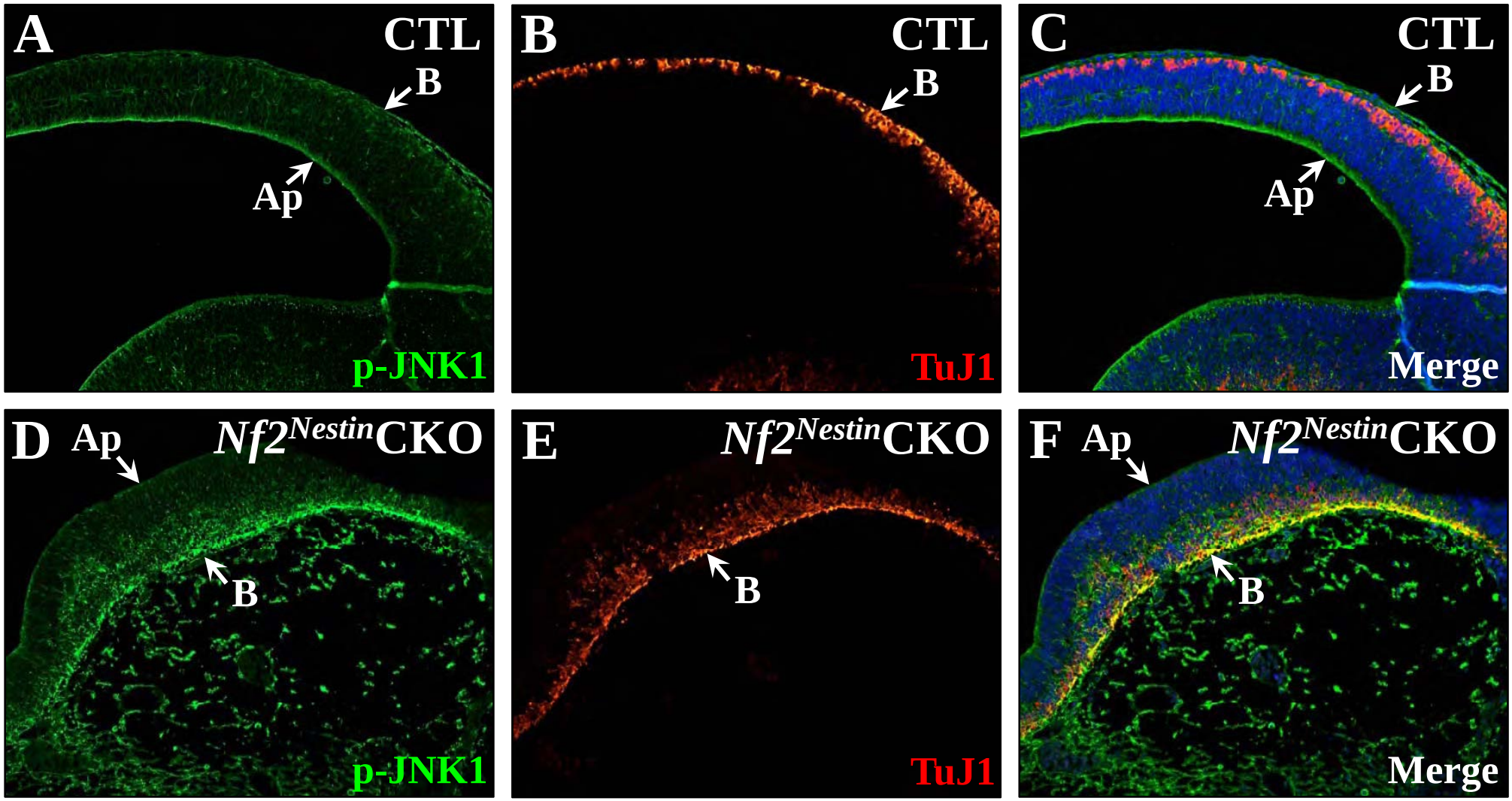

A
CTL
Ap
N-cadherin
B
Nf2NestinCKO
Ap
N-cadherin
C
CTL
Ap
β-catenin
D
Nf2NestinCKO
Ap
β-catenin

Figure 6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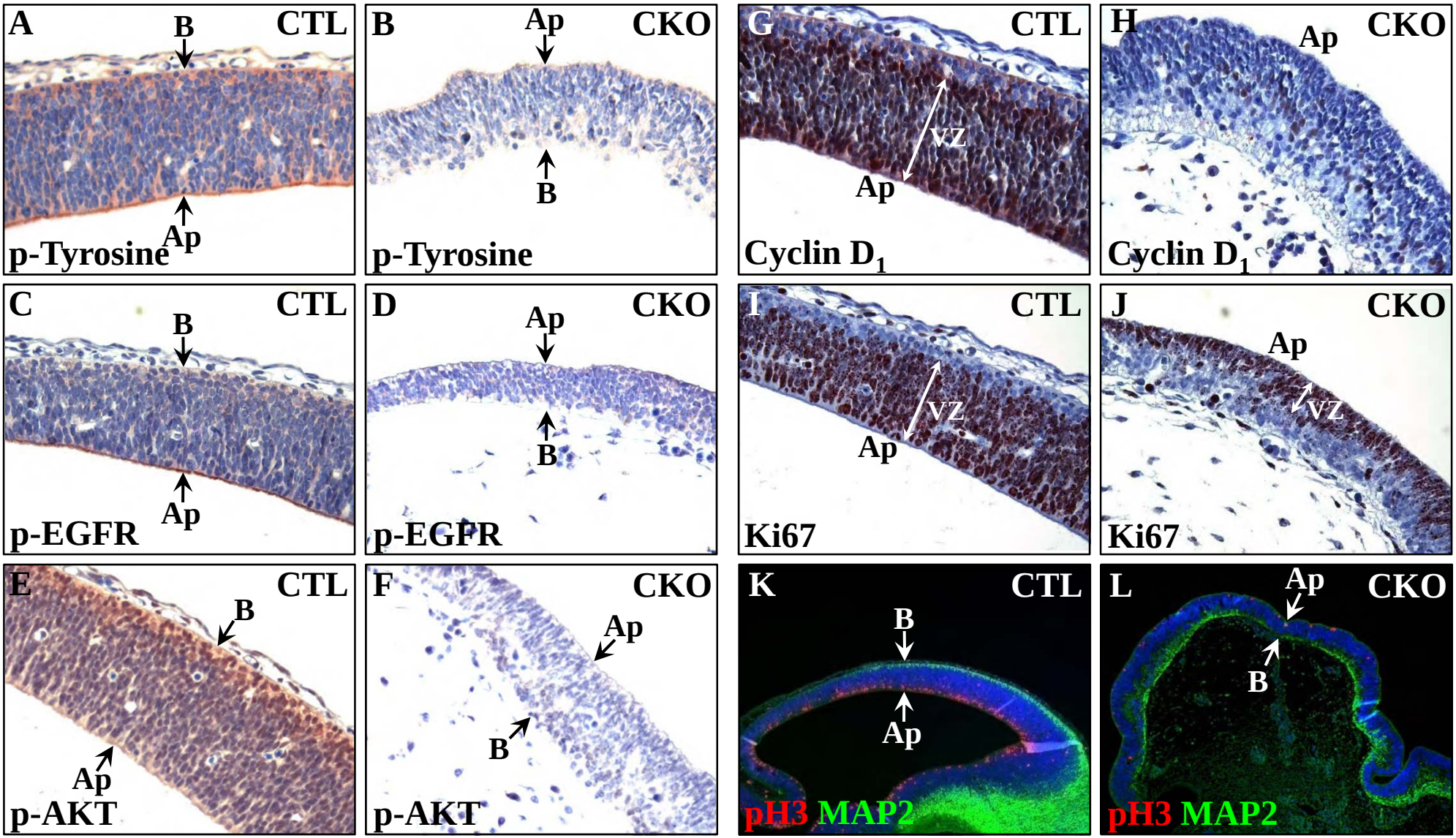
B
Ap
p-JNK1
B
CTL
B
TuJ1
C
CTL
B
Ap
Merge
D
Ap
Nf2NestinCKO
B
p-JNK1
E
Nf2NestinCKO
B
TuJ1
F
Ap
Nf2NestinCKO
B
Merge

Figure 7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CTL
B
Ap
p-Tyrosine
B
CKO
Ap
B
p-Tyrosine
G
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VZ
Ap
Cyclin D1
H
CKO
Ap
Cyclin D1
C
CTL
B
Ap
p-EGFR
D
CKO
Ap
B
p-EGFR
I
CTL
VZ
Ap
Ki67
J
CKO
Ap
VZ
Ki67
E
CTL
B
Ap
p-AKT
F
CKO
Ap
B
p-AKT
K
CTL
B
Ap
pH3 MAP2
L
CKO
Ap
B
pH3 MAP2

Figure 8

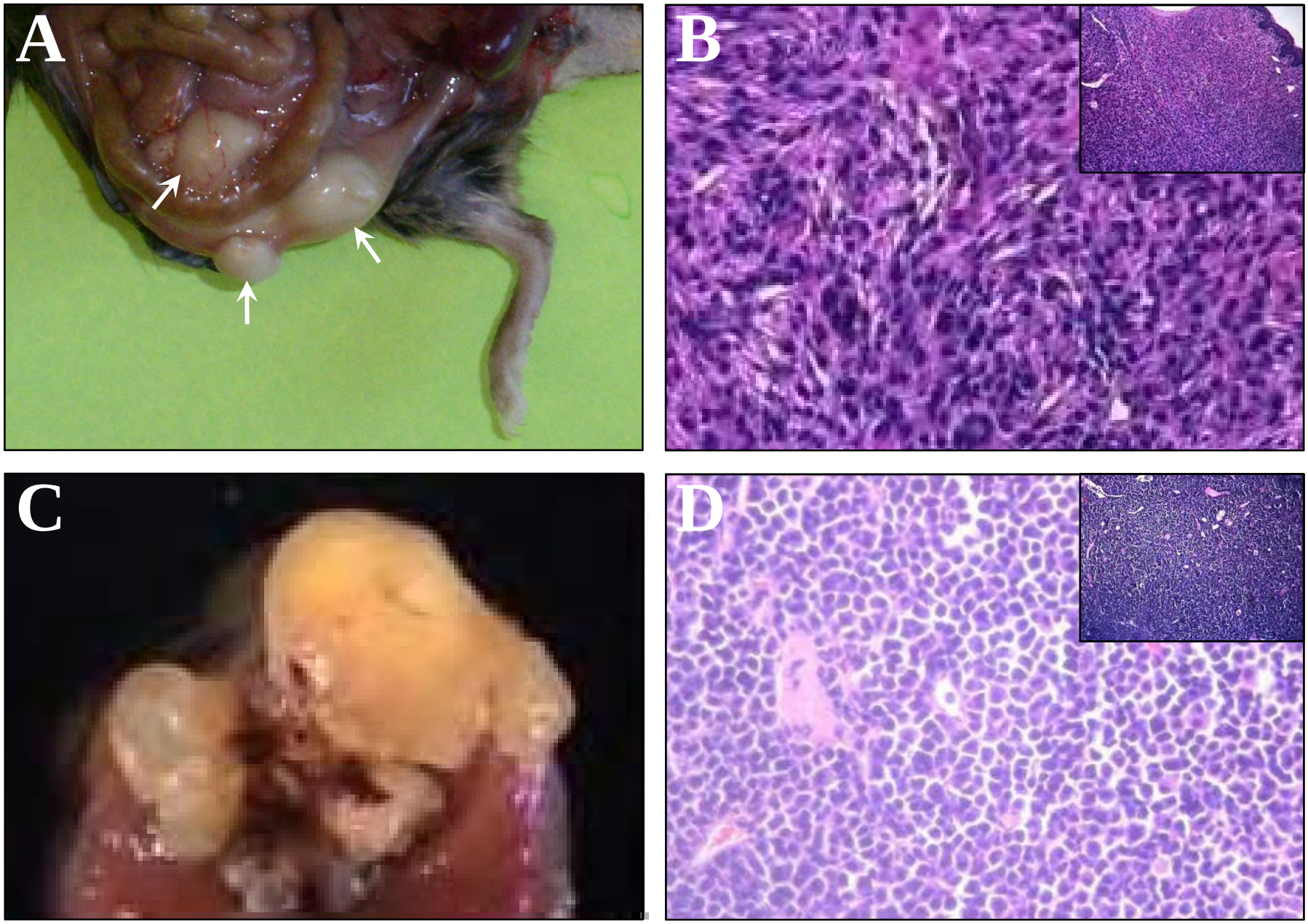


Figure 8 (Cont'd)

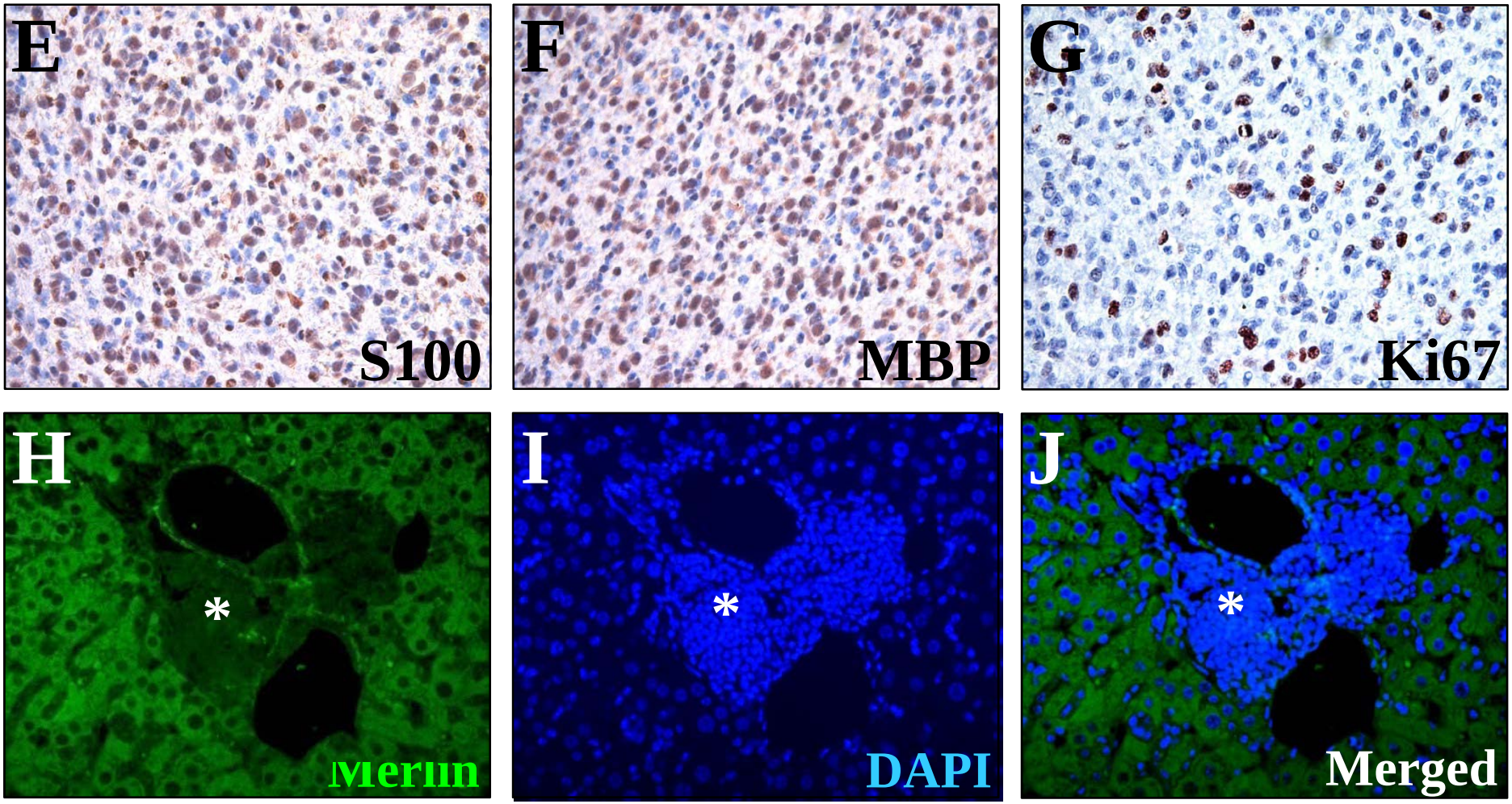


Figure 9A

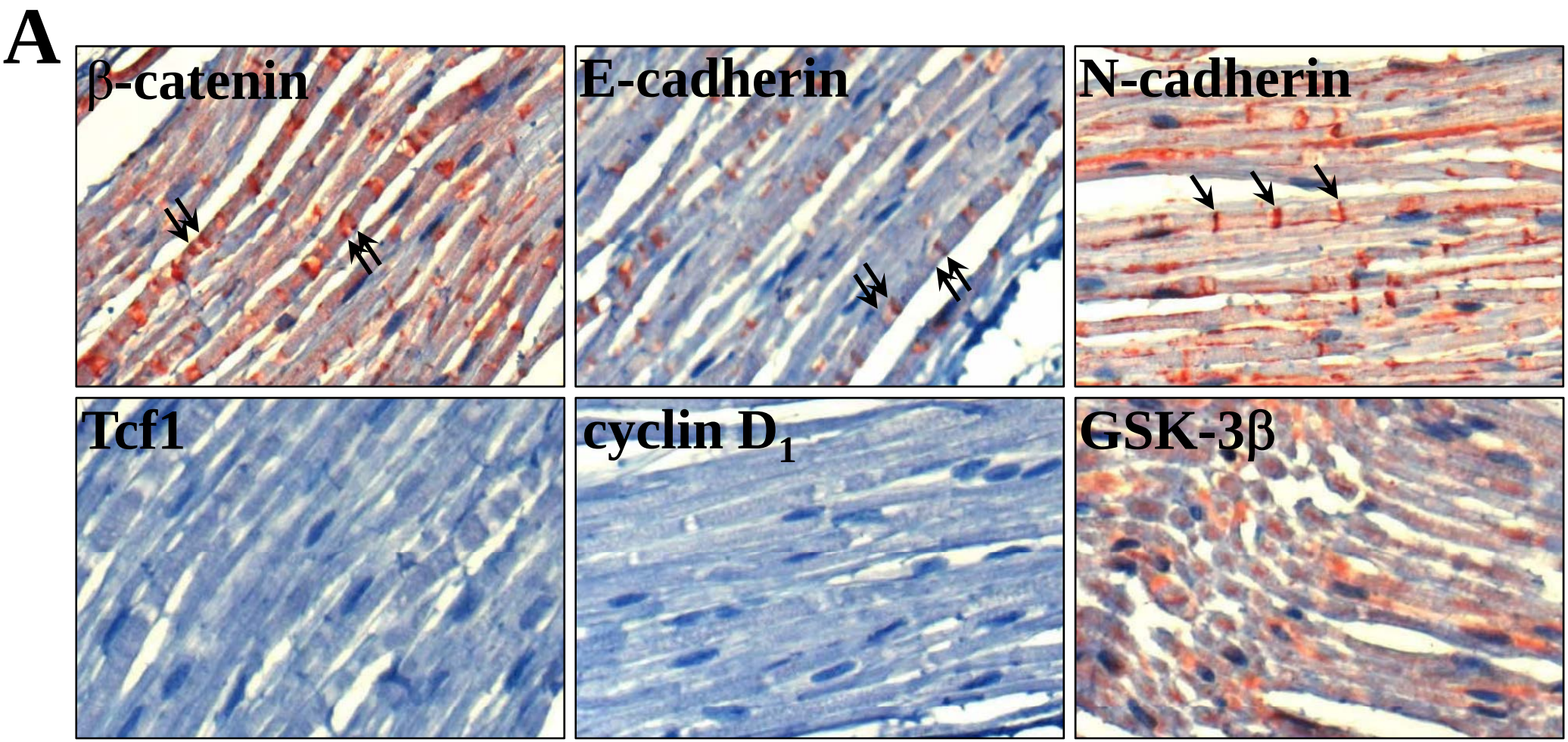


Figure 9B

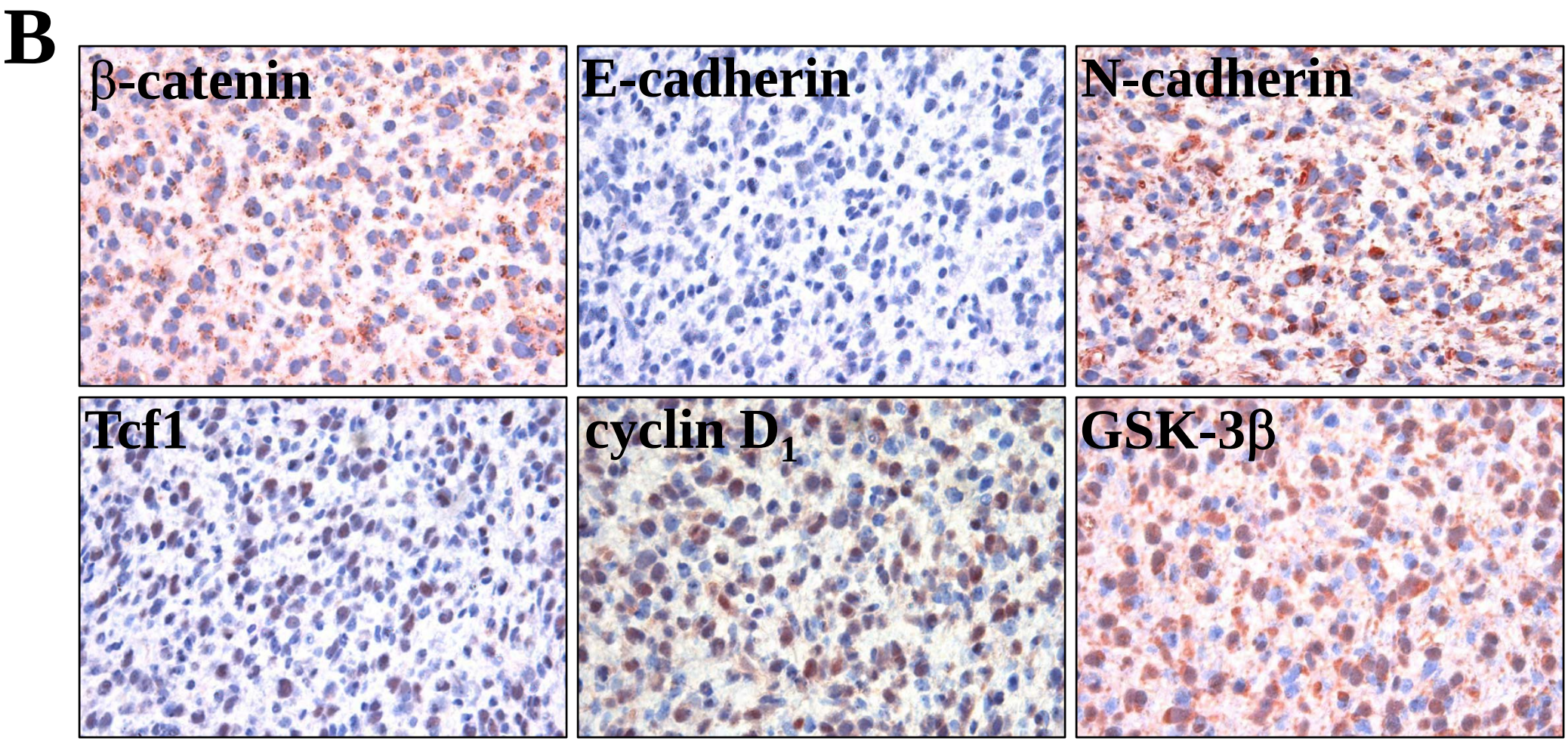


Figure 9C

C

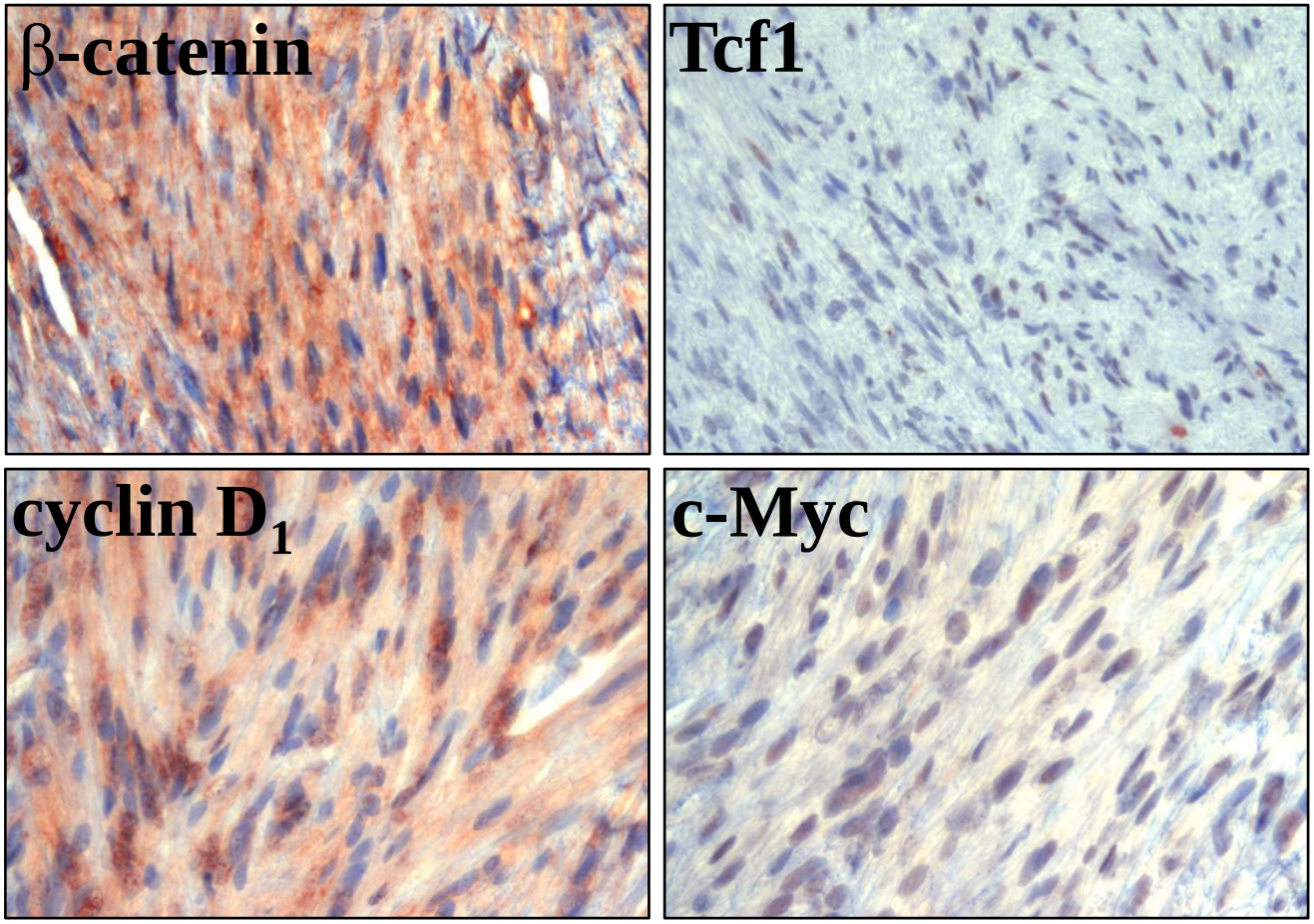


Figure 10A

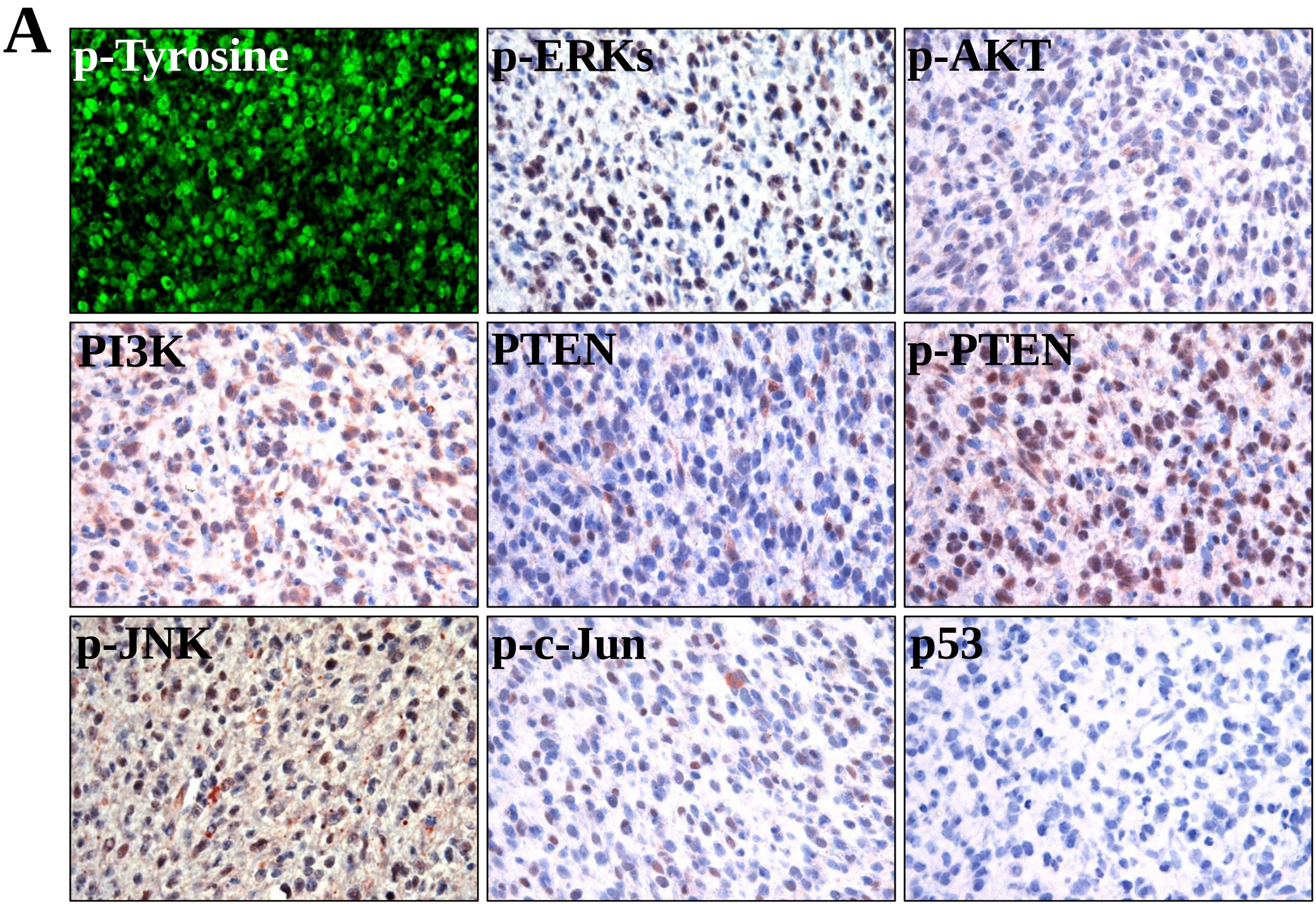


Figure 10B

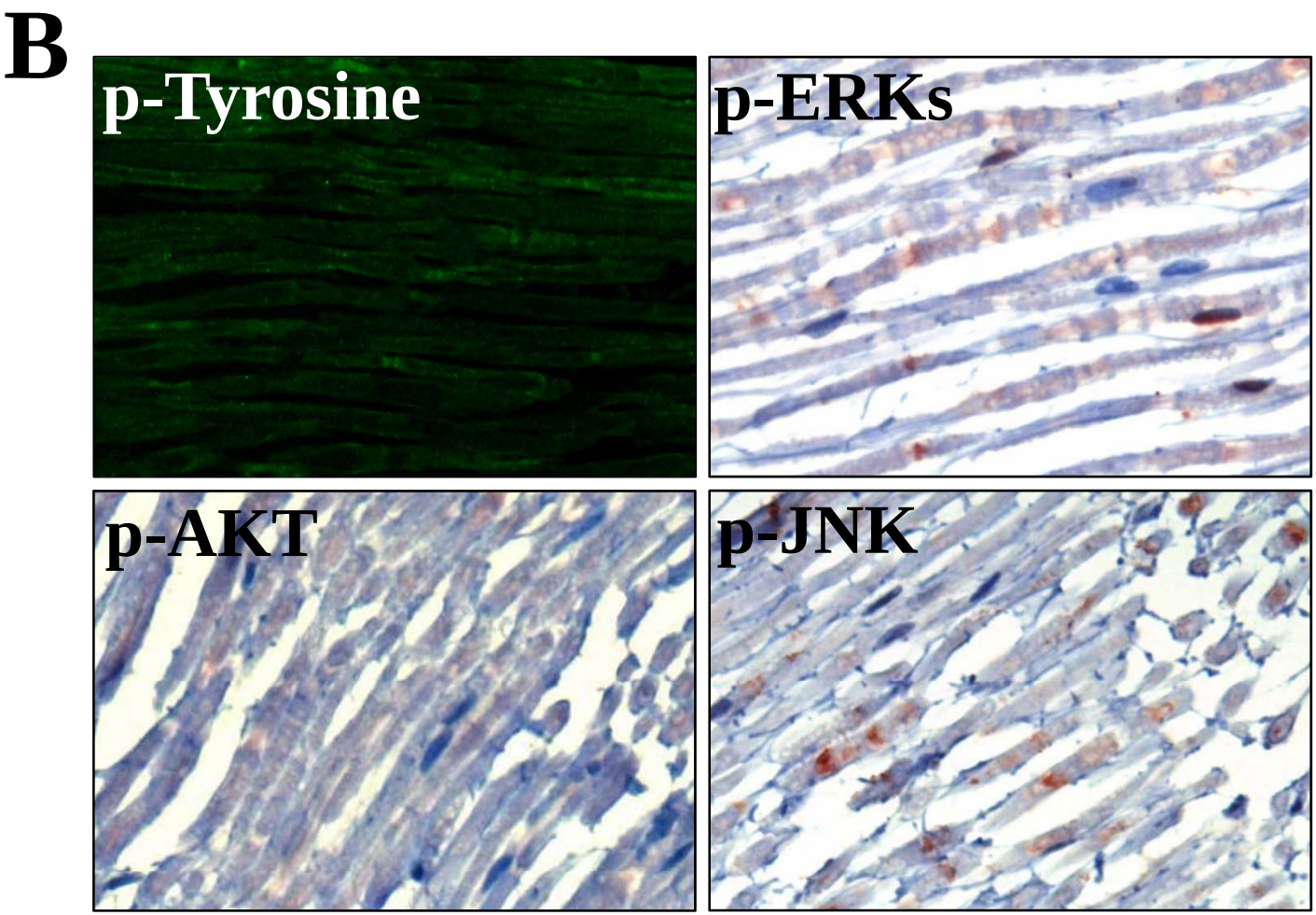
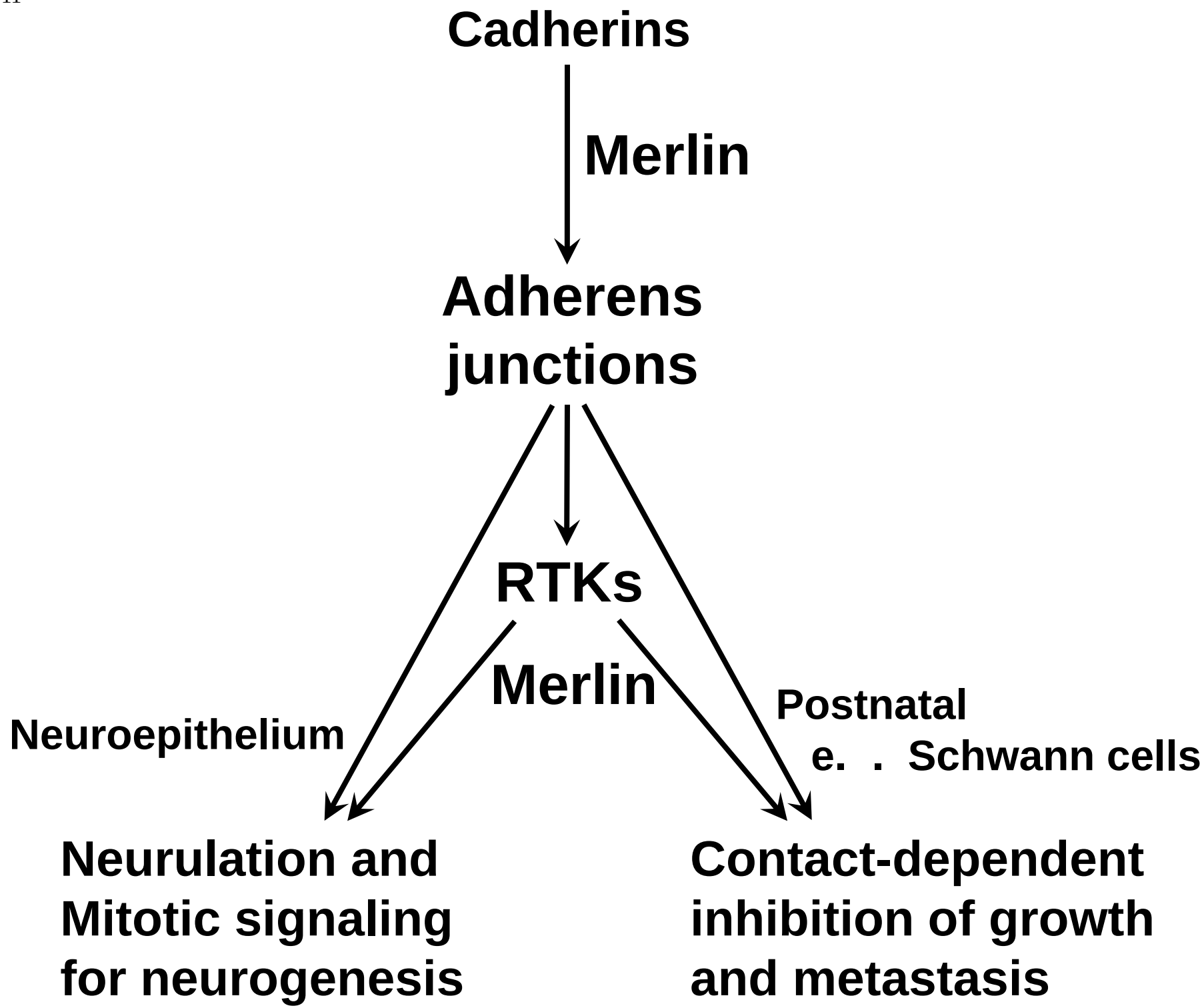
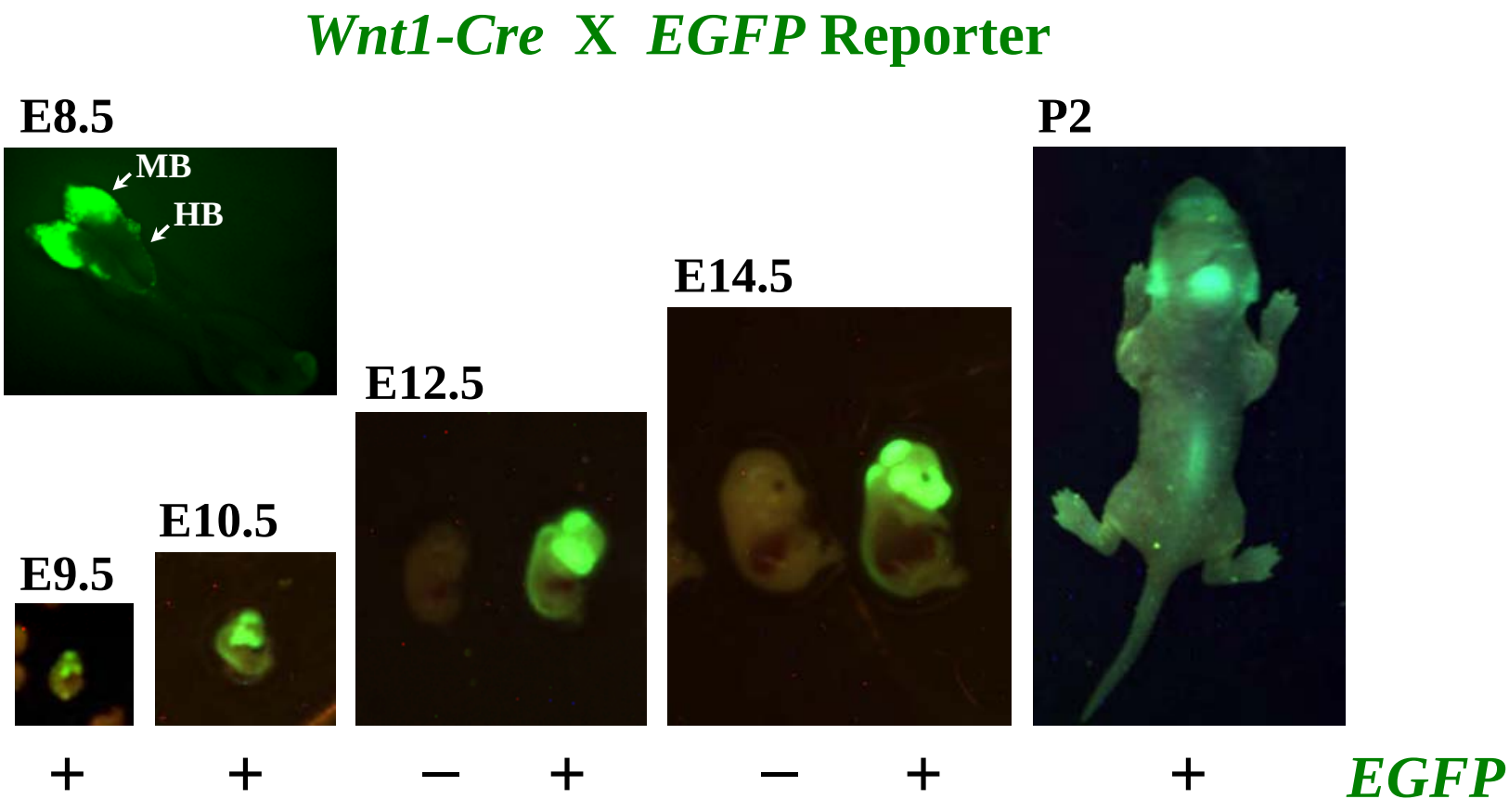


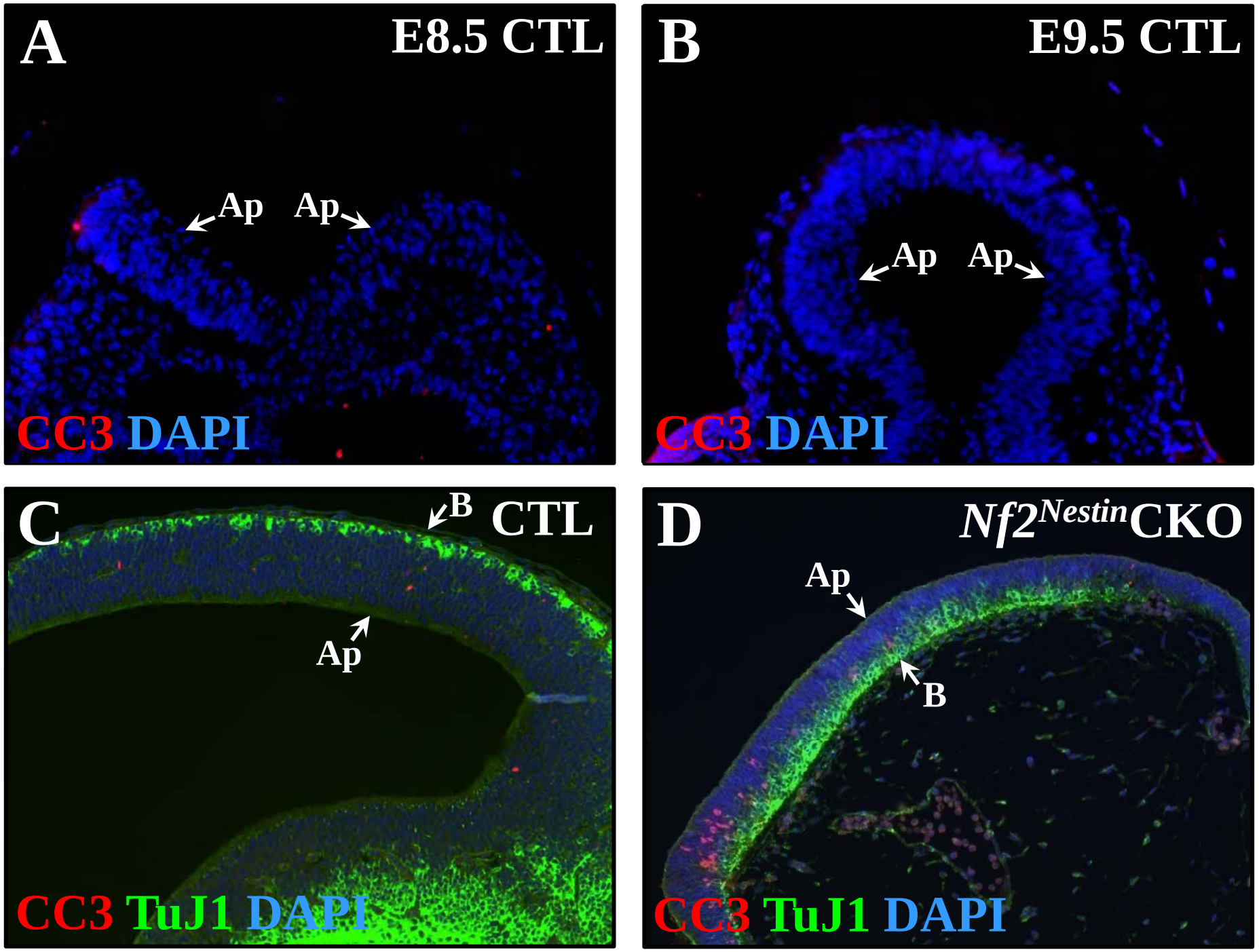
Figure 11


Cadherins
Merlin
Adherens junctions
RTKs
Merlin
Neuroepithelium
Postnatal
e. . Schwann cells
Neurulation and Mitotic signaling for neurogenesis
Contact-dependent inhibition of growth and metastasis

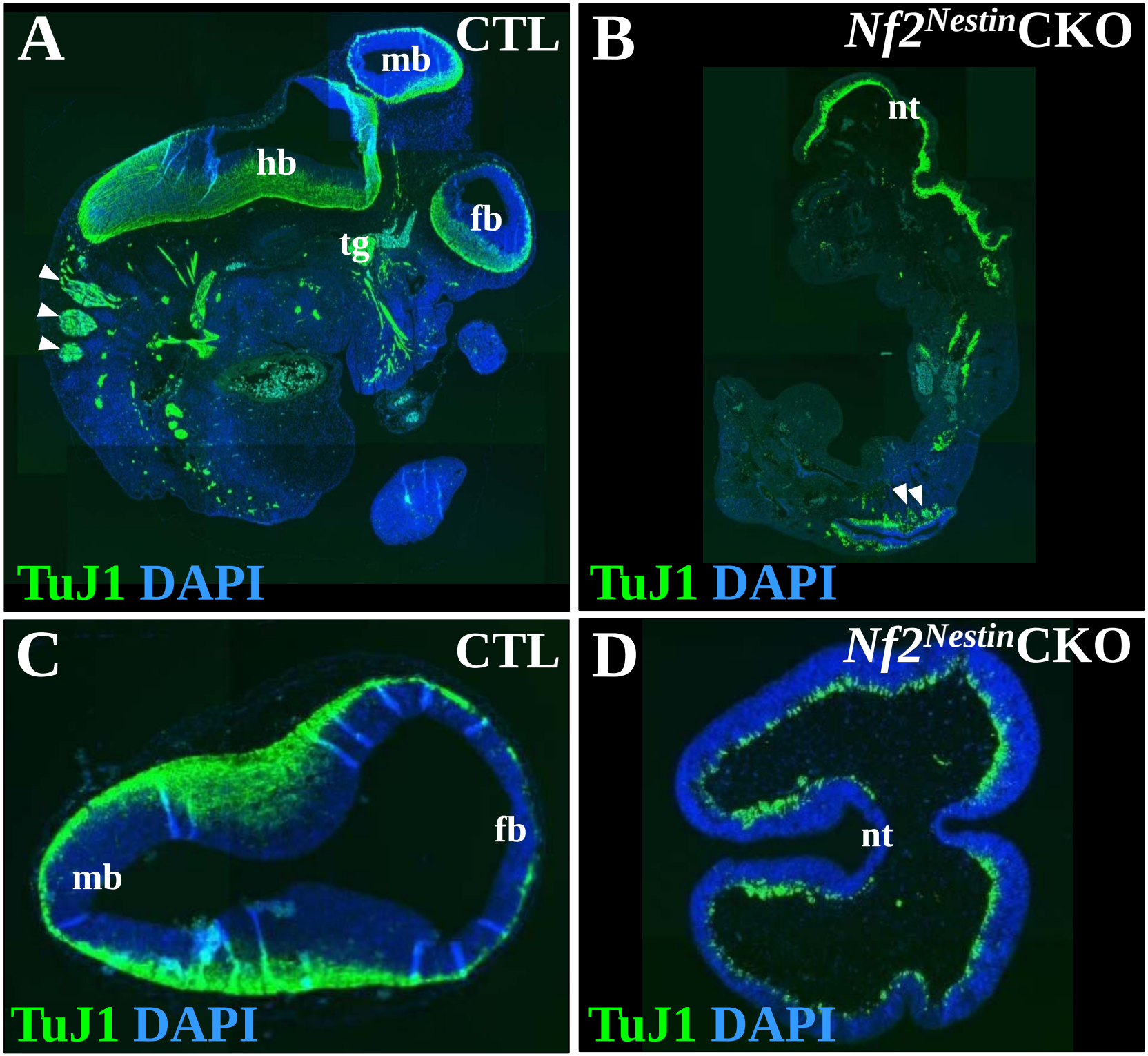
Supplemental Fig. S1



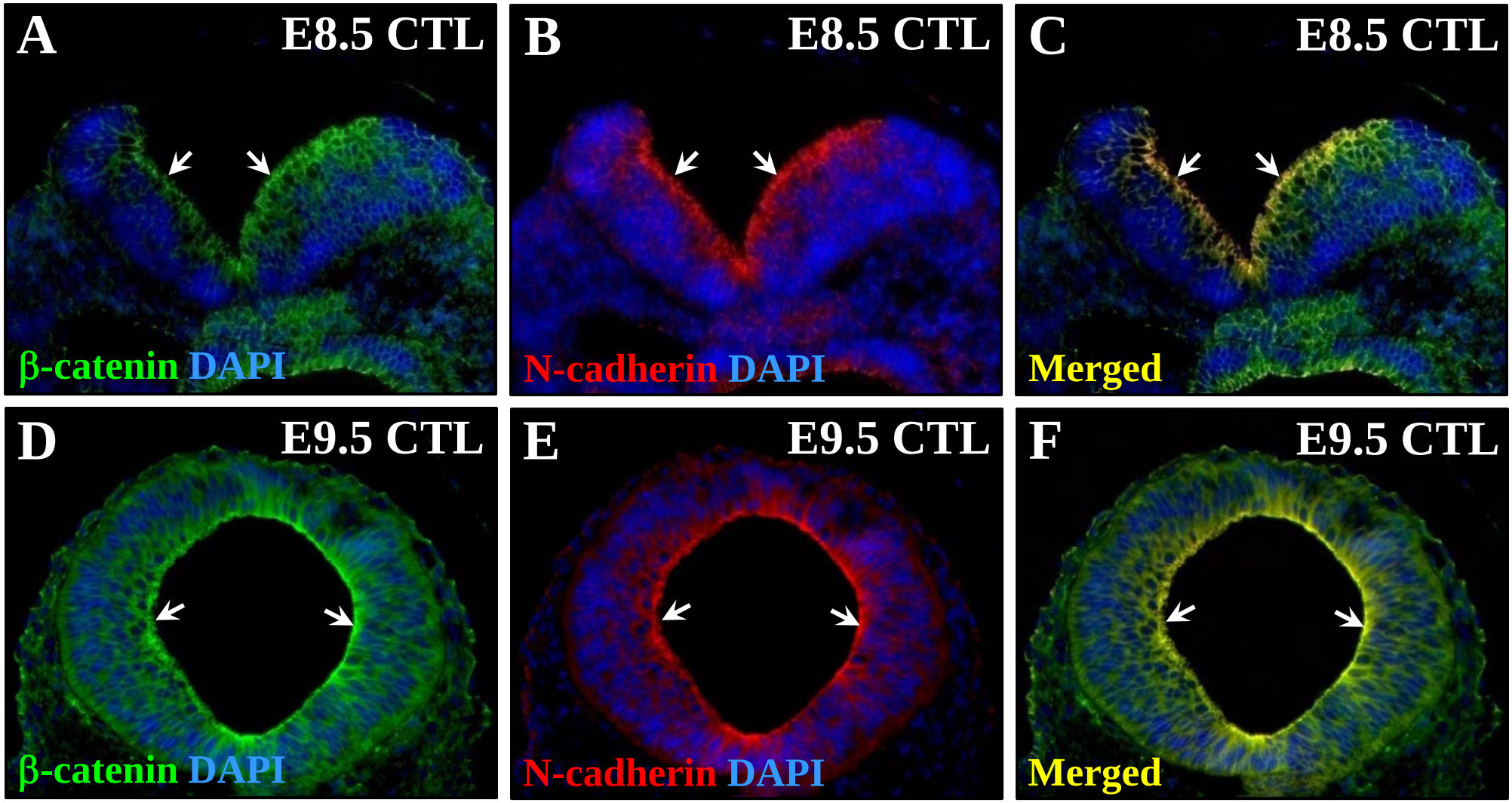
Supplemental Fig. S2



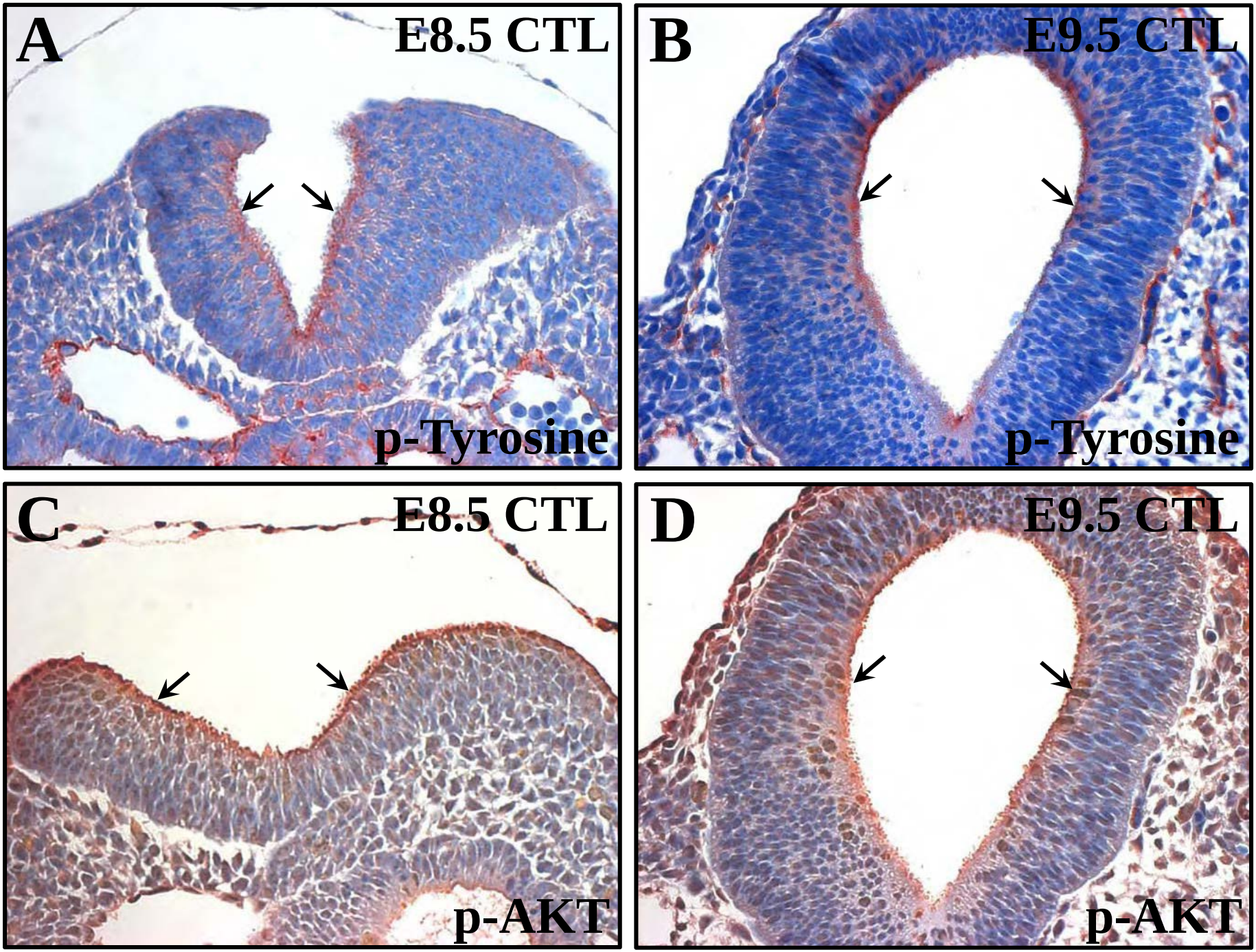
Supplemental Fig. S3



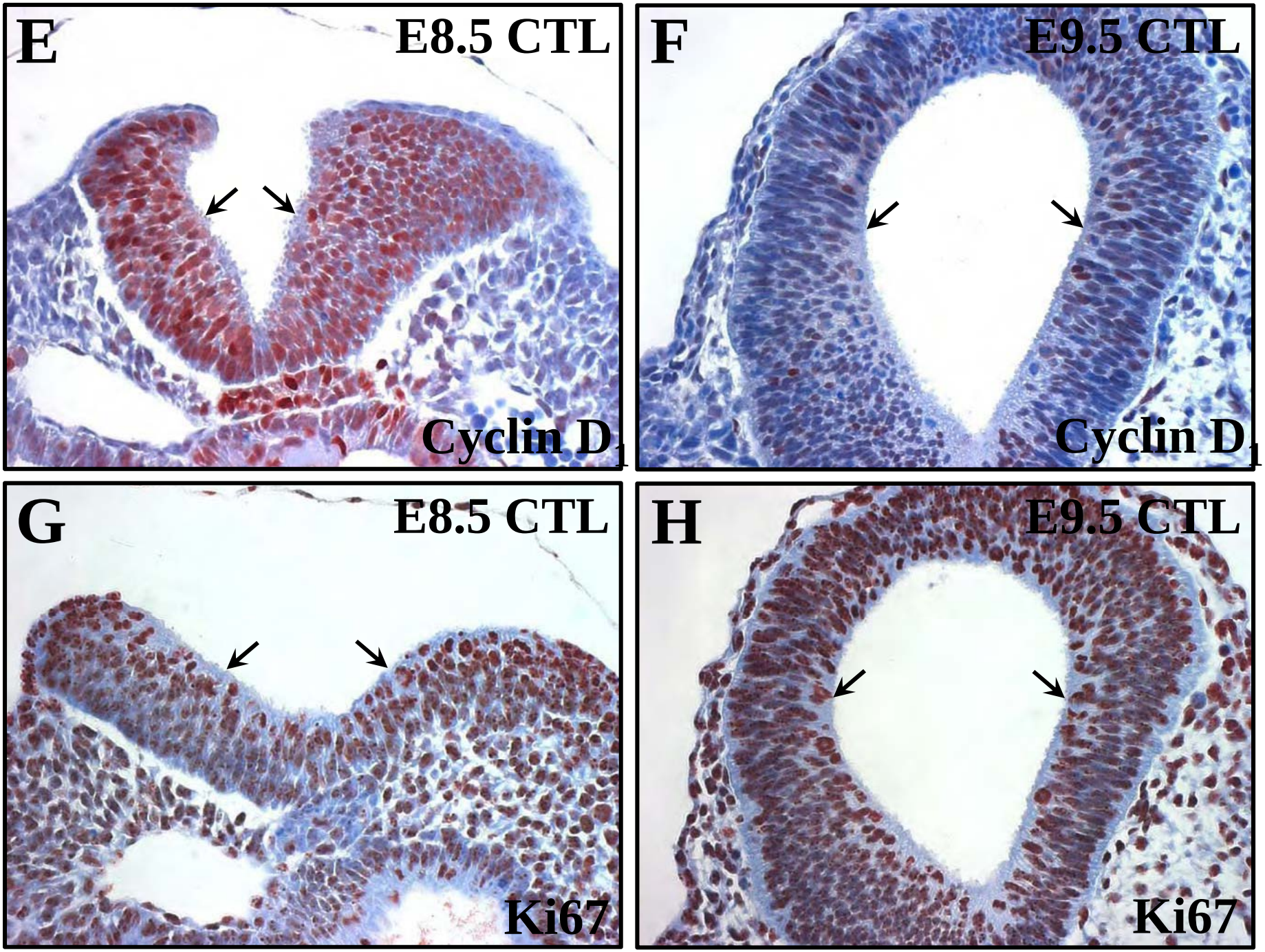
Supplemental Fig. S4



Supplemental Fig. S5A-D



Supplemental Fig. S5E-H



Supplemental Fig. S6